STUDIES OF SERUM AND ERYTHROCYTE MEMBRANE PROTEINS IN CYSTIC FIBROSIS AND DUCHENNE MUSCULAR DYSTROPHY

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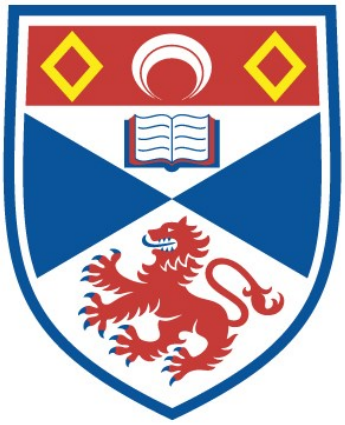

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STUDIES OF SERUM AND ERYTHROCYTE MEMBRANE PROTEINS IN CYSTIC FIBROSIS AND DUCHENNE MUSCULAR DYSTROPHY

by I.M. ZINZOMBE

A thesis submitted to the
University of St. Andrews in application
for a degree of Doctor of Philosophy

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SUMMARY

(I) Recent research on Duchenne muscular dystrophy lends support to the notion that the genetic defect may affect the surface membranes of not only muscle cells but other cells particularly erythrocytes. Abnormalities in the content and compositions of fatty acids and phosphorylations of some erythrocyte membrane proteins have been reported in support of this hypothesis. SDS gel electrophoresis patterns of erythrocyte membrane proteins from Duchenne muscular dystrophy were reported to be normal. In this report, SDS gel electrophoresis of erythrocyte membrane proteins prepared by hypotononic lysis of red blood cells at pH8, were not able to show any differences between samples from Duchenne muscular dystrophy patients and those from normal control subjects. The relative intensities of the protein bands varied from preparation to preparation but did not follow a pattern that could distinguish control samples from test samples. Two dimensional gel electrophoresis of completely denatured erythrocyte membrane proteins resolved over 150 peptides in 9% gels and over 200 peptides in gels above 12%, but no consistent differences were observed between Duchenne muscular dystrophy samples and those from control subjects.

(II) Although cystic fibrosis is the most frequent lethal genetic syndrome among caucasian children, there is no known biochemical or structural defect to account for all its pathophysiological phenomena. Previous reports in other laboratories have shown that a cystic fibrosis protein (CFP) could be demonstrated in sera of over 90% of cystic fibrosis homozygotes by isoelectric focusing. The protein was characterised as having a molecular weight of between 3000-10 000 and pI near pH8.4. Our isoelectric focusing results demonstrated the protein, pI8.5, in 40% of the homozygotes, thus supporting the view

that the procedure is not a useful diagnostic tool and that the "cystic fibrosis protein" is not a specific marker for the disorder. We extended this investigation by labelling, with ^{125}I, all the serum proteins that focused between pH8 and pH9 and then running the labelled proteins in some 15% SDS gels. The results we obtained could not distinguish cystic fibrosis samples from normal control ones.

Some studies have suggested that a mutant form of alpha-2-macroglobulin could be the primary defect in cystic fibrosis and also in multiple sclerosis. To test for the presence of charge or size mutations, alpha-2-macroglobulin was subjected to extensive investigations by (i) SDS gel electrophoresis of its heat fragments, (ii) two dimensional gel electrophoresis following complete denaturation and (iii) immuno-electrofocusing of its native form. Alpha-2-macroglobulin from cystic fibrosis patients could not be distinguished from that obtained from normal control subjects but that from multiple sclerosis patients showed some different patterns. The significance of the abnormality of α_2M from multiple sclerosis patients needs further investigations.

Extensive two dimensional gel electrophoresis of whole serum following complete denaturation were performed but the results did not reveal any consistent differences between cystic fibrosis and multiple sclerosis patients and normal control subjects.

DECLARATION

I hereby declare that the following thesis is based on work carried out by me, that the thesis is my own composition and that no part of it has been presented previously for a higher degree.

The research was carried out in the Department of Biochemistry and Microbiology of the University of St. Andrews under the direction of Dr. M.G. Burdon.

CERTIFICATE

I hereby declare that Ignatius M. Zinzombe has spent nine terms in research work under my supervision and that he has fulfilled the conditions of Ordinance No.16 (St. Andrews) and that he is qualified to submit this thesis for the degree of Doctor of Philosophy.

ACKNOWLEDGEMENTS

I would like to thank my Supervisor, Dr. M.G. Burdon, for his encouragement and advice throughout this work; the British Council and the Cystic Fibrosis Research Trust for their financial support; Mr. Bill Blythe for all the photographic work; technical staff for their assistance; friends for donating blood and Mrs. Cooper for typing this thesis.

ACADEMIC RECORD

I matriculated at the University of Reading in September, 1975 and graduated with an Honours degree of Bachelor of Science, lower Second Class in Physiology and Biochemistry in July 1978.

In October 1978, I matriculated as a research student at the University of St. Andrews.

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I MUSCULAR DYSTROPHY

1. GENERAL

"DYSTROPHY" literally means 'inadequately nourished', from the Greek: DYS - mis, TREPHEIN - to nourish.

The muscular dystrophies have been defined as heterogenous group of disorders consisting of a few of the inherited myopathies (myopathy - non-neurogenic neuromuscular disorder). Not all diseases bearing the name 'muscular dystrophy' are inherited e.g. vitamin E deficiency, neither are all diseases causing muscle wasting and weaknesses called muscular dystrophies. Maltase deficiency, for example, causes muscle wasting but it is not classified as a dystrophy. In this report, discussions of muscular dystrophies will be limited to primary, genetically determined, progressive and degenerative myopathies. The basic muscle defect in many of these conditions is an abnormality in muscle fibre membranes (Rowland, 1976; Schotland et al., 1977; Carpenter S. et al., 1979) but the primary biochemical mechanism producing this alteration is still unknown. Laboratory findings have reported increased activities of serum enzymes such as creatine kinase, adenylate kinase, pyruvate kinase; histologic alterations on muscle biopsy; altered properties of red cell membranes; disturbances in ribosomal activities and abnormal electromyographic recordings.

2. CLASSIFICATION OF MUSCULAR DYSTROPHIES

Muscular dystrophies may be classified as proposed and modified by Walton and Gardner-Medwin (1974) into

(i) autosomal dominant

(ii) autosomal recessive

and (iii) X-linked recessive conditions as shown in Table 1.

In autosomal dominant inheritance, an individual need have only one gene of a given type to be affected. An affected individual has an affected parent except in the case of new mutation or incomplete penetrance.

In autosomal recessive inheritance, an affected individual has the given gene in double dose. Relatives at risk are generally siblings. For offspring of two carriers, the probability is $^1/_4$ for being affected and $^1/_2$ for being a carrier.

In X-linked recessive inheritance, an affected individual has no normal X-chromosome. The great majority of affected individuals are males. Affected individuals of the same family are related through females. A female carrier married to a normal male has, in a given pregnancy, an equal chance of producing an affected male, normal male, a carrier female and a normal female.

TABLE 1

CLASSIFICATION OF MUSCULAR DYSTROPHIES

(i) X-linked recessive forms:

Duchenne muscular dystrophy

Becker-type muscular dystrophy

Mabry's et al. variant of muscular dystrophy

Emery and Dreifuss variant of muscular dystrophy

(ii) X-linked dominant forms:

Muscular dystrophy confined to females

(iii) Autosomal recessive forms:

Limb girdle muscular dystrophies

Childhood muscular dystrophies

Congenital muscular dystrophies

Quadriceps myopathy

(iv) Autosomal dominant forms:

Facioscapulohumeral muscular dystrophy

Distal muscular dystrophy

Ocular muscular dystrophy

Oculopharyngeal muscular dystrophy

Proximal muscular dystrophy

Myotonic muscular dystrophy

2.1 Duchenne muscular dystrophy

Although Duchenne is generally given the credit for describing the first case of muscular dystrophy in 1861, it was Meryon in 1852 who initially characterised muscular dystrophy and suggested that it was a primary muscle disorder.

Duchenne or pseudohypertrophic dystrophy is a progressive muscular dystrophy inherited as an X-linked recessive trait. Expression of this syndrome occurs only in males but inheritance is through the female line. The prevalence of this syndrome is nearly 3 in 100 000 (Gardner-Medwin, 1970) and one-third of these cases are believed to be new mutants (Haldane, 1935; Zatz et al., 1977). The onset of the disease begins in the first 3 to 5 years of life and is characterised by weakness of the lower limbs and pelvic girdle musculature. In the early stages of the disease, muscle hypertrophy occurs, usually in the calves, and hence the term 'pseudohypertrophic dystrophy'. In the early stages of the disease, dystrophic muscle is capable of regenerating spontaneously (Pearson, 1962; Hudgson et al., 1967; Mastaglia et al., 1969) and Satellite cells are markedly increased in numbers and they show features suggesting that they are activated for protein synthesis (Wakayama et al., 1979). As the disease progresses, the hypertrophic muscles degenerate and are replaced by fat and fibrous tissue (Emery, 1977). Later shoulder girdle becomes involved and in the more advanced stages virtually all the voluntary muscles of the body are atrophied and weak. The relentless progression of the disease leads to the inability to walk within ten years and death from inanition, respiratory infection and cardiac failure, usually in the second decade.

In the initial biochemical studies, there were no specific clues to the nature of the genetic abnormality. There was no observed accumulation

of an abnormal metabolite to point to the defective enzyme and there was no overt abnormality of metabolic patterns within muscle.
One of the earliest biochemical observations made in Duchenne muscular dystrophy was the increased excretion of creatine. Soon after, Sibley and Lehninger (1949) were attempting to evaluate the use of serum aldolase assay as a screening test for malignancy when they observed elevated aldolase activity in two Duchenne dystrophy boys who were among their controls. Subsequently, a host of other enzymes including lactate dehydrogenase, glutamate-oxaloacetate transaminase, phosphoglucomutase, glycerophosphoisomerase, creatine phosphokinase (Ebashi et al., 1959) and pyruvate kinase (Harano et al., 1973; Alberts et al., 1974) were found to be elevated in Duchenne dystrophy serum. Although not proved directly, it was assumed that the enzyme activities in sera of affected patients were derived mostly from muscle sarcoplasm. This was suggested because the enzymes involved were, without exception, decreased in muscle sarcoplasm (Somer et al., 1973) whereas membrane-bound enzymes did not appear in the serum. One interpretation of the increased serum enzymes suggests that there is a genetic fault of the muscle membranes leading to the leakage of the sarcoplasmic enzymes.

The diagnosis of Duchenne muscular dystrophy may be confirmed by estimating serum creatine phosphokinase, which is confined mostly to muscle and central nervous system, and pruvate kinase activities and by muscle biopsy. The estimation of the enzyme activities have proved to be the most sensitive assays in detecting patients and carriers of Duchenne muscular dystrophy. Serum creatine kinase levels are highest early in the disease and decline as the condition progresses (Pearce et al., 1964). Electrophoretic studies have identified serum creatine kinase as being of the MB type, an isoenzyme predominant in cardiac muscle (Goedde et al., 1978) and pyruvate kinase as being of the M_1 type (Harano et al.,

1973, Zatz M. et al., 1978).

At present there is no effective treatment of the disease. In general practice, a woman who is found to be at high risk of having an affected son can elect to have amniocentesis with antenatal fetal sexing and selective abortion of any male fetuses. The major drawback to this method is that a proportion of such aborted fetuses will be normal.

2.2 Becker-Type X-linked muscular dystrophy

Becker (1955) was the first to draw attention to the existence of a benign form of X-linked muscular dystrophy. The onset of the disease begins in the first decade or early in the second (mean age = 11 years). Affected individuals do not become confined to wheelchairs until twenty to thirty years after the onset. The pattern of muscular weakness is similar to that seen in Duchenne muscular dystrophy but the progression is much slower than in the Duchenne variety. The condition is less common than the Duchenne variety with a prevalence of 1 per 100 000. Serum creatine phosphokinase activity is substantially elevated in affected boys. Like the Duchenne variety, hypertrophy affects the calves but this may precede other symptoms by some years. Fifty to sixty per cent of the carriers have increased creatine phosphokinase activities and these decrease significantly with increasing age (Skinner R. et al., 1975). In isolated cases, patients with early onset of the disease may be difficult to distinguish from Duchenne dystrophy patients. The EMG and muscle biopsy may demonstrate fibrillation potentials, positive waves and occasional high frequency discharges as well as polyphasic motor unit potentials of short duration (Pearn and Hudgson, 1978). Muscle biopsies reveal prominent hypertrophic fibres as well as random atrophy and

splitting of fibres and central nuclei and fibrosis.

Affected individuals may reproduce, although their fertility is somewhat reduced. The males will transmit the abnormal gene to all their daughters who will be carriers. The males will be non-affected if the mother has normal X-chromosomes.

Two forms of benign X-linked muscular dystrophy have been described which apparently seem to be distinct from the Becker type. Mabry et al. (1965) described a type which appears to differ from the Becker type by the age of onset and the great disability which it produces. The Emery et al. type (1966) differs from the Becker type by the absence of hypertrophy of the calves and by the development of contractures at the age of 4 to 5 years. Evidence of myocardial involvement is apparent. In the second decade of life serum creative kinase activity is 6 to 7 times the normal value, but subsequently it gradually falls with increasing age.

2.3 Limb-girdle muscular dystrophy

Limb-girdle muscular dystrophy is inherited as an autosomal recessive trait in about 60 per cent of the cases and the rest are believed to be sporadic. Both sexes are equally affected. This form of muscular dystrophy is characterised by variable age of onset, but usually occurs in the second or less often in the third decade of life. The condition progresses slowly but constantly. Symptoms are similar to those in Duchenne type but with a more benign course. Severe disability is usually present 20 years after the onset when the hip flexors and quadriceps muscles become affected. Musculature involvement may start either in the shoulder or pelvic girdle. Hypertrophy is not a feature and contractures are not a major problem. The heart is normal and intelligence is

unimpaired. Serum creatine phosphokinase is elevated in most cases although much less so than in X-linked forms. Electromyography and muscle biopsy findings are typical of myopathic processes.

Recent and careful investigations suggest that most cases of limb-girdle are either

(i) males with Becker dystrophy

(ii) manifesting female carriers of the Duchenne gene

(iii) patients with spinal muscular atrophies

or (iv) patients with a variety of acquired myopathies including carnitine deficiency, acid maltase deficiency and polymyositis, (Moser and Emery, 1974).

2.4 Fascioscapulohumeral muscular dystrophy

Fascioscapulohumeral muscular dystrophy is inherited as an autosomal dominant trait with a wide variation of expression. It is relatively uncommon with a prevalence of about 0.2 to 0.5 per 100 000. Some patients have only slight weaknesses of closure of eyes whilst some occassional patients are handicapped within the first 2 years of life and may be unable to walk before the age of 20 years. In most patients, fascioscapulohumeral muscular dystrophy begins with weakness in the facial and shoulder girdle muscles usually by the end of the second decade. The patients are characterised by facial weakness which leads to inability to close their eyes and to whistle and in more advanced cases speech may become indistinct. The heart is rarely affected and the intellect is almost always normal.

Serum creatine kinase activity may be raised but rarely to 5 times the normal value, but about 50 per cent of the patients have normal levels of the enzyme. Pyruvate kinase is raised in most patients and it

could be a better adjunct for confirming the diagnosis of the disease. Muscle biopsis reveal inflammatory infiltrates. Milder cases of fascioscapulohumeral muscular dystrophy may resemble those of the Driefuss type of muscular dystrophy, myotonic dystrophy, mitochondrial myopathies, myasthenia gravis, polymyositis or scapuloperitoneal syndrome.

3. ERYTHROCYTE MEMBRANES

3.1 General

Classical observations led to the theory that the plasma membrane was a thin layer of lipoid material interspersed with minute water-filled channels. Later, chemical analyses of plasma membranes indicated that they were composed predominantly of lipid and protein with small amounts of carbohydrates. Subsequently it was suggested that the plasma membranes consisted of a bimolecular lipoid sheet bound on both sides by protein. Recent studies have put forward a more dynamic model in which lipids and intrinsic and extrinsic proteins are viewed as being organised in a mosaic structure. The intrinsic (integral) proteins are embedded or intercalated in disordered arrangement into a discontinuous lipid bilayer that forms the matrix of the mosaic. The intrinsic proteins are bimodal with their ionic and highly polar groups located largely on the membrane surfaces in contact with the extracellular and intracellular aqueous media and with the non-polar residues in the lipid bilayer. The extrinsic proteins, which constitute the bulk of the proteins, are located exclusively at the cytoplasmic side of the membrane external to the lipid bilayer. These are bound to the cytoplasmic surface of the membrane by electrostatic and hydrophobic interactions.

In general, the most elementary membranes contain the least protein while those membranes exhibiting the highest functional complexity contain the most protein. Many of the basic physical properties common to all cell membranes appear to be a reflection of their predominantly lipid nature.

3.2 Erythrocyte membrane lipids

Lipids of red blood cell membranes are organised as one double layer of closely packed molecules - the lipid bilayer. Most of the membrane lipids are phospholipids which contain two hydrophobic hydrocarbon chains extending in one direction from a glycerol backbone and a hydrophilic head group extending in the opposite direction. This structural organisation produces an intrinsically amphipathic molecule whose dual physical properties are of fundamental importance in bilayer formation and maintenance.

In erythrocytes and indeed most mammalian plasma membranes, individual lipid molecules in either half of the bilayer have a high degree of lateral mobility at physiological temperature. On the other hand, movement of phospholipid molecules from one half of the bilayer to the other ("flip-flop" transitions) is largely restricted presumably by the unfavourable energetic considerations entailed in moving the charged hydrophilic group through the hydrophobic membrane interior. The flip-flop transitions occur at rates several times slower than lateral translations within the plane of the membrane.

In membranes with heterogenous phospholipid composition, lipids with similar temperature-dependent acyl chain properties may segregate into discrete lateral microdomains within the membrane (Wu S.H. and McConnell H, 1974). Some membrane proteins selectively partition into or become functional in certain of these microdomains, therefore the lipid

segregation may be of considerable importance in the control of membrane behaviour.

Erythrocyte membranes show lipid asymmetry when the phospholipids of the inner or cytoplasmic half of the bilayer are compared with those of the outer or extracellular half. Two thirds of the choline-containing phospholipids and about 80 per cent of sphingomyelin are located on the outer half of the bilayer. The remaining major phospholipid components, phosphatidyl ethanol amine and phosphatidyl-serine are located mainly in the inner half of the bilayer. The remaining other major component, cholesterol, is present in patches between the bilayer halves. Cholesterol modifies fluidity of the membranes.

3.3 Erythrocyte membrane proteins

3.3.1 General

Erythrocyte membranes contain eight major polypeptide chains when analysed by sodium dodecyl sulphate (SDS) gels using the procedure and nomenclature of Fairbanks et al. (1971). When periodic acid-Schiff's reagent (PAS) a carbohydrate stain sensitive to the presence of sialic acid, is applied to SDS gels of total membrane proteins, four prominent bands designated PAS-1, PAS-2, PAS-3 and PAS-4 become evident. The bulk of the major proteins (bands 1, 2, 4, 5 and 6) are located exclusively at the cytoplasmic side of the membrane, external to the lipid bilayer. This was confirmed by labelling of inside-out membrane vesicles (Berg H. et al., 1969; Bretscher M., 1971) and proteolytic cleavage experiments (Bender W. et al., 1971; Steck T et al., 1971; Triplett et al., 1972). These extrinsic proteins could be isolated from membranes by simple means such as manipulation of ionic strength or pH. Extrinsic proteins comprise

about 40 per cent of the total membrane protein. No major membrane protein has been found located exclusively at the outer surface of the membrane and no protein completely buried within the bilayer.

A second class of proteins can be removed from the membrane only by rigorous treatment with detergents. Such treatment results in the dissolution of the membrane structure. Proteins that require these methods are called intrinsic membrane proteins. The two main integral proteins are (i) glycophorin and (b) polypeptide band-3. Intrinsic proteins are held in place in the membrane by hydrophobic interactions between non-polar surface amino acid residues of the protein and the hydrophobic hydrocarbon core of the membrane. These membrane proteins are glycoproteins and they are oriented so that those portions containing sugar residues are exposed on the extracellular half of the membrane.

3.3.2 Extrinsic membrane proteins

Extrinsic membrane proteins comprise of bands 1 and 2 (spectrin), 2.1, 2.2 (ankyrin), 4 (uncharacterised), 5(actin) and 6 (glyceraldehyde-3-phosphate dehydrogenase).

(a) Spectrin

Spectrin (Marchesi et al., 1968) comprise 20-30 per cent of the total erythrocyte membrane proteins. It is extracted in water-soluble form by exposing membranes to low ionic strength buffers containing EDTA at 37°C. It is conceived that spectrin is present as a two dimensional network which interacts loosely with the over-lying lipid bilayer. The submembraneous localisation of spectrin in intact human red cells has recently been confirmed by using ferritin-conjugated anti-spectrin antibodies and electron microscopy (Ziparo E. et al., 1978).

Suggested functions of spectrin include determining of shape of red blood cells, stabilising of lipid bilayer, contribution to lipid asymmetry and influencing distribution of integral membrane proteins. The human erythrocyte spectrin molecule is a tetramer composed of two copies of two different polypeptide chains with an aggregate molecular weight of about 900 000. The individual polypeptide chains of spectrin have been referred to as bands 1 and 2. Band 1 chain has an apparent molecular weight of 240 000 and band 2 has molecular weight of 200 000. Band 2 polypeptide is phosphorylated in intact red cells. In vitro, the dimeric form of spectrin predominantes at 37°C and the tetrameric at 4°C with the transition temperature at 28°C (Ungewickel E. et al., 1978). Spectrin tetramer can bind to the F-form of actin but not the G-form (Brenner et al., 1979) and it is associated with actin (band 5) in intact erythrocytes. When red cell ghosts were suspended in Triton X-100, the bulk of the membrane lipids and all integral proteins were lost into the medium. The residue, consisting mainly of spectrin and actin could be examined by scanning electron microscope. A reticular pattern was observed (Hainfield et al., 1977).

Spectrin is now known to exist in close proximity to band 3 and band 4.1. Ankyrin (band 2.1) is implicated in binding spectrin subunits to band 3, 4.1 and 4.2 (Bennet et al., 1979).

(b) Actin (Band 5)

Band 5 migrates on SDS polyacrylamide gels with an apparent molecular weight of 42 000 (Fairbanks et al., 1971). It is attached on the cytoplasmic side of the membrane and coextracts with spectrin and some minor proteins (Puszkin et al., 1978). The red blood cell actin, like the skeletal muscle actin, activates myosin Mg-ATPase, interacts with troponin and tropomyosin to confer calcium sensitivity to red blood cell

MgATPase. Whilst muscle actin activates Mg-ATPase optimally at 25°C, that of red blood cells requires 37°C for optimal activity. Polymerisation of actin is controlled by the phosphorylated state of spectrin (Pinder et al., 1977).

The rest of the extrinsic membrane proteins are comprised of band 4 which has not yet been characterised and band 6 (glyceraldehyde-3-phosphate dehydrogenase).

3.3.3 Integral membrane proteins

Integral membrane proteins are comprised mainly of band 3 and glycophorins (PAS-1, PAS-2 and PAS-3).

(a) Band 3

Band 3 is the broad Coomassie blue-stained area that migrates with an apparent molecular weight of 90 000 - 100 000. It is composed of a number of heterogenous molecules that have similar apparent molecular weights on sodium dodecyl sulphate gels. The sum of these heterogenous array of molecules constitutes about 30 per cent, by weight, of the membrane proteins (Bretscher, 1971). Some of the band 3 molecules like the acyl-phosphorylated polypeptide are associated with Na, K, Mg-ATPase activity, while others, like the intrinsic (integral) glycoprotein have been linked to anion transport. The glycoprotein comprises 8-10 per cent of the exterior surface glycoprotein.

Recent experiments have demonstrated that the amino-terminal end of band 3 glycoprotein is on the cytoplasmic side of the membrane and the carboxyl terminal is on the external surface of the membrane. The carbohydrates are located near the carboxyl terminal end, outside the membrane. Preliminary work indicates that the size of the protein buried inside the lipid core of the membrane which is inaccessible to

to proteases from inside or outside the membrane amounts to about 20 per cent by weight. Chymotrypsin treatment of ghosts results in the cleavage of a fragment of molecular weight 30 000 from the amino terminal end. Purified band 3 has been found to contain about 10 per cent carbohydrates, mainly mannose, galactose and N-acetyl glucosamine in approximate ratios 1:2:2 and traces of fucose and glucose. The carbohydrate is believed to be linked N-glycosidically to asparaginyl residues. The movement of band 3 and possibly of other transmembrane proteins seems restricted by a linkage of some kind to the peripheral protein spectrin, apparently via mutual interactions with a third protein referred to as ankyrin (band 2.1 mainly).

(b) Sialoglycoproteins

These are visualised on sodium dodecyl sulphate gels by periodic acid-Schiff's reagent, a carbohydrate stain sensitive to the presence of sialic acid. The main sialoglycoprotein, glycophorin, contains carbohydrates amounting to 60 per cent of its dry weight. Seventy-five per cent of the total sialoglycopeptides is constituted by glycophorin A, the basic subunit of PAS-1. Glycophorin A, has a molecular weight of 31 000 with a polypeptide chain containing 131 amino acids. Most of the polypeptide mass lies in the cytoplasmic side and a very small portion of the amino acid residues is exposed on the external surface of the membrane. Of the 131 amino acid residues, 72 residues at the NH_2-terminal end are located on the external side of the membrane and a sequence of 23 predominantly apolar, hydrophobic residues form the intramembrane and transmembrane portion. The 36 amino acids at the carboxyl-terminal end are located on the cytoplasmic side of the membrane. All sugar residues are extracellular and are attached to the amino residues on the outside of the membrane.

On SDS gels, glycophorin A is visualised as two bands PAS-1 and PAS-2. It is now known that the two polypeptides are interconvertible and that PAS-1 is a dimer of PAS-2. Glycophorin B, on the other hand, is an oligomeric series which comigrate with PAS-1, PAS-2, PAS-3 and PAS-4. The structure of glycophorin B is similar to that of glycophorin A but the C-terminal peptide exposed on the cytosolic side in glycophorin A is missing in glycophorin-B. It is believed that the absence of an extended cytoplasmic polypeptide in glycophorin B allows this peptide to undergo unrestricted translational motion.

4. MEMBRANE DEFECTS IN MUSCULAR DYSTROPHIES

4.1 General

The very first biochemical abnormality to be recognised in Duchenne muscular dystrophy was the increased excretion of creatine. This phenomenon was later observed in other wasting conditions, but subsequent kinetic studies with carbon-14 creatine (Fitch et al., 1968) suggested that whilst there was decreased transport of creatine into muscle in other wasting conditions, there was impaired trapping of intracellular creatine in dystrophic muscle. This observation suggested that the muscle membranes were abnormal or leaky in Duchenne muscular dystrophy. Later and more recent experiments have found many enzymes including aldolase, creatine phosphokinase, transaminase, lactate dehydrogenase, adenylate kinase and pyruvate kinase to be elevated in serum in dystrophic patients. Leaky muscle membranes have been incriminated for such increases.

It is an established fact that genetic biochemical abnormalities may be found in cells other than those of symptomatic tissues. In

muscular dystrophies, investigations have been directed to cells other than muscle cells particularly erythrocytes and fibroblasts. Other cells have received attention in these disorders because biochemical studies on muscle membranes are thwarted by practical problems of isolating pure muscle cells and membranes. Fibroblasts have been used in biochemical investigations because of the relative ease with which they could be cultured in vitro. Erythrocytes have received attention because they are readily available and their membranes can be obtained in pure form. Some workers in this field consider the approach of using erythrocytes as misdirected because the erythrocyte is a specialised cell unable to perform many biochemical reactions such as protein synthesis.

4.2 Structural changes in muscle cell membranes

In Duchenne muscular dystrophy, muscle fibre necrosis is seen from an early age, even before the disease becomes clinically manifest (Pearson C.M., 1962; Hudgson P., et al., 1967). Whether or not it begins in the neonatal period or during intra-uterine life is not clear, although activities of creatine kinase in the blood may be considerably elevated at these stages (Mahoney et al., 1977). Some investigators have suggested that focal ischaemia may be responsible for muscle necrosis. This suggestion arose from the observations of groupings of necrotic and regenerating fibres (Hathaway et al., 1970; Scarlato G. et al., 1977) in muscles. Considerable evidence which has accumulated from morphological studies over the years suggests that a structural or functional abnormality of the plasma membrane may be the basis for the fibre necrosis. In 1975, Mokri and Engel reported in muscle fibres of Duchenne dystrophy patients the appearance of small focal wedge-shaped zones of contraction and wedged and rounded zones of peroxidase infusion

into the edges of muscle fibres and major breaks of the plasmalemma in similar regions evident in high resolution phase electron microscopy. These lesions were considered to be disease specific and to provide evidence of a primary plasmalemma defect. Some electron microscopical observations (Carpenter and Karpati 1979) provided evidence suggesting some repair by muscle fibre of small areas of plasma membrane breakdown.

Further evidence for the abnormality of sarcolemma membrane in dystrophic muscle has been provided by freeze-fracture preparations of skeletal muscle. In Duchenne dystrophy, an abnormal non-uniform distribution of intramembranous particles and a depletion of their number on both the protoplasmic and extracellular faces of the muscle membranes has been observed (Schotland et al., 1977; Schotland et al., 1980). In myotonic dystrophy, such particles were markedly increased. They suggested that the observed depletion and altered distribution of intramembraneous particles in Duchenne dystrophy might be an early sign of membrane abnormality that precedes the formation of focal defects at the cell surface membrane. Recently Bonilla et al., (1978) have reported focal defects in the binding of concanavalin A to the plasma membrane of muscle cells. There is ample evidence suggesting that the defective muscle fibre plasmalemma allows excessive quantities of Ca^{2+} to accumulate within the dystrophic muscle. Such increases in Ca^{2+} have been demonstrated by using histochemical (Bodensteiner J. et al., 1978) and electron-cytochemical techniques (Oberc and Engel 1977).

4.3 Structural changes in erythrocyte membranes

Erythrocytes in carriers and patients of Duchenne dystrophy have been reported as being abnormal in the form of "echinocytes" (crenated forms) (Matheson D. et al., 1974; Grassi et al., 1978). However this

observation was not disease specific, having been reported in limb-girdle dystrophy and in mouse muscular dystrophy (Morse and Howland 1973). In 1976, Miller and co-workers reported an abnormally high incidence of cup-shaped erythrocytes, "stomatocytes" when studied by scanning electron microscope. It is perceived that such observed changes might result from some kind of membrane defect. It should, perhaps, be mentioned here that studies have shown that some agents could cause crenation and others cupping of erythrocytes. Crenating agents are normally cationic. Drugs such as barbiturates and salicyclates and depletion of ATP lead to crenation and local anaesthetics lead to cupping of erythrocytes. Some observers believe that the changes in form of erythrocytes from dystrophic patients could have resulted from the therapy received by these patients.

Freeze-fracture preparations of erythrocyte membranes from Duchenne dystrophy patients indicated a decreased and non-uniform distribution of intramembraneous particles (Wakayama et al., 1979) on both the protoplasmic and extracellular faces of the membrane. These observations were identical to those observed in muscle cell membrane.

Circumstantial experimental evidence suggests that Ca^{2+} may be increased in erythrocytes of Duchenne dystrophy patients. Dise C.A. et al., (1977) observed that the changes in normal erythrocytes as a consequence of increased intracellular Ca^{2+}, induced by ionophore A 23187, were identical to those observed in Duchenne dystrophy. They suggested that the abnormalities in erythrocyte membrane properties in Duchenne dystrophy might result from a defect in the regulation of membrane function by Ca^{2+}. In an attempt to find the factors regulating the intracellular content of Ca^{2+}, Plishker and Appel (1980) measured in vitro the uptake of Ca^{2+} by erythrocytes from Duchenne muscular dystrophy patients. They

observed no difference in uptake between these and erythrocytes from control subjects.

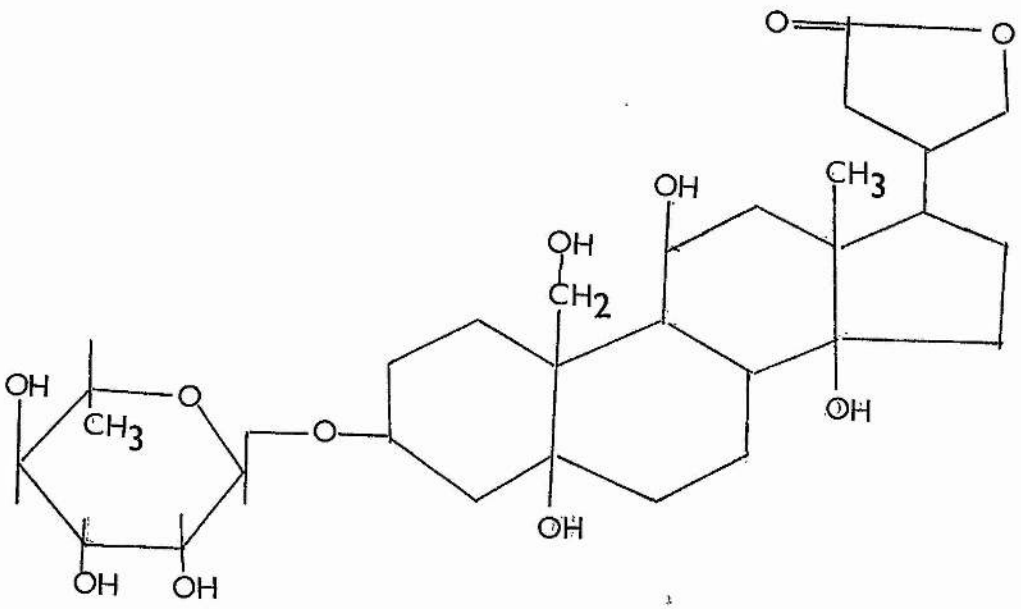
Investigators on membrane properties in muscular dystrophy have also directed their attention to their physical properties. Butterfield et al. (1976) using 2-(3 carboxypropyl)2-tridecyl-4,4-dimethyl-3 oxazolindinyl methyl ester, commonly called 5-nitroxide methyl stearate (5NMS), spin label demonstrated that erythrocytes membranes from myotonic dystrophy and congenital myotonia patients were more fluid than controls, and those from Duchenne dystrophy patients were less fluid. They also reported a correlation between increased erythrocyte membrane fluidity and myotonia. Subsequent experiments using methyl-5-nitrosostearate spin label (Wilkerson et al., 1978) demonstrated increased rigidity of erythrocyte membranes from Duchenne dystrophy patients. They suggested that the observations could be a reflection of increased rigidity of membrane proteins in dystrophic erythrocytes. These observations were consistent with Percy and Miller (1975)'s report of reduced deformability of erythrocytes from Duchenne muscular patients.

4.4 Abnormalities in membrane-bound enzymes

The activities of various enzymes such as aldolase, creatine kinase, and pyruvate kinase are highly increased in sera of Duchenne dystrophy patients. It is conceived that these enzymes egress from muscle sarcoplasm into the circulation. This suggestion stems from the observation that these enzymes are without exception normally found in the sarcoplasm and that membrane-bound enzymes are not found increased in the serum of the same patients. These observations have been attributed to a leaky muscle membrane. In an attempt to test the "leaky membrane hypothesis" some investigators have directed their research to enzymes associated with

sarcolemma particularly the $(Na^+ + K^+)$ ATPase.

The $(Na^+ + K^+)$ ATPase is an active transport system which creates a substantial gradient of Na^+ and K^+ across the cell membrane. The gradient is achieved by maintaining a very high extracellular Na^+ and very high intracellular K^+ concentration (Figure 1). The $(Na^+ + K^+)$ ATPase requires Mg^{2+} for maximum activity and is normally inhibited by ouabain, a cardiac glycoside whose structure is shown below.



OUABAIN

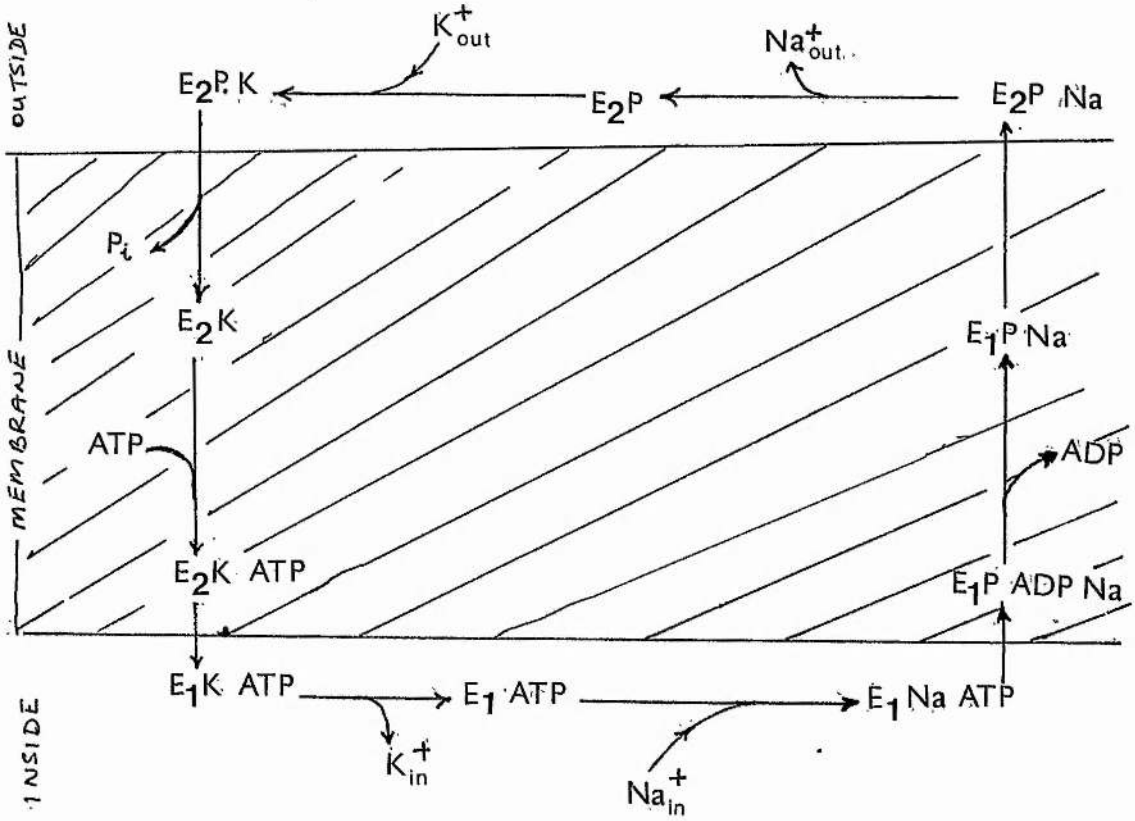
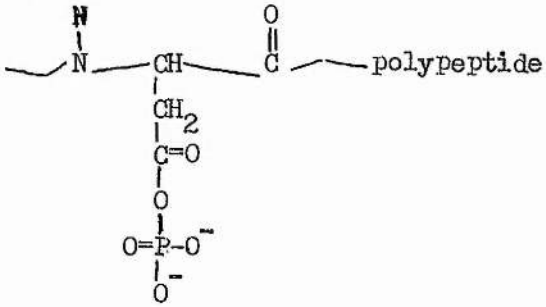


Figure 1 Postulated mechanism of transport of $(Na^+ + K^+)$ ATPase.

Aspartyl side chain of enzyme is phosphorylated as:



Adapted from Karlish et al., B.B.A. 525 (1978) 252.

Though the studies of $(Na^+ + K^+)$ATPase in whole muscle extracts were frustrated by all other ATPases present in the muscle, Dhalla et al. (1973) reported a decreased activity of the enzyme in the sarcolemma from Duchenne dystrophic patients. Experiments with erythrocyte $(Na^+ + K^+)$ATPase were more productive. Brown et al (1967) reported that the $(Na^+ + K^+)$ATPase of erythrocyte ghosts which is normally inhibited by ouabain, was stimulated in erythrocytes from Duchenne dystrophy, myotonic dystrophy and congenital myotonia patients. The observation was later disputed by Klassen et al. (1969) but subsequent experiments substantiated Brown and coworkers' findings (Araki S. et al., 1971). Soon after, Hanahan D.J. (1973) observed that the levels of enzymes in isolated erythrocyte membranes varied with conditions of red cell storage, lysis and washing. Recently, Hanahan and Ekhom (1978) have clearly illustrated that enzyme activities varied with the method of ghost preparation. Table II over, adapted from Hanahan and Ekholm (1978) illustrates the variability of $(Na^+ + K^+)$ATPase and $(Ca^{2+} + Mg^{2+})$ATPase activities in erythrocyte membranes prepared from same donors using different methods of cell lysis but same assay conditions. The data illustrate the difficulties encountered in comparing membrane-bound enzymatic assays from different laboratories. It is possible that the conflicting data on ATPase activities, as alluded to above, may be due to variabilities on techniques used.

TABLE II

VARIABILITY OF $(Ca^{2+} + Mg^{2+})$ AND $(Na^{+} + K^{+})$ MgATPase LEVELS PREPARED BY DIFFERENT HEMOLYSIS PROCEDURE

PROCEDURE		$(Ca^{2+} + Mg^{2+})$ ATPase	$(Na^{+} + K^{+})$ ATPase
I	HYPOTONIC HEMOLYSIS (20 mOsmol)		
	NaCl histidine pH 7.6	12	3
	TRIS-HCl pH 7.6	20	10
	TRIS-maleate pH 5.8	12	3
II	HYPOTONIC HEMOLYSIS (20 mOsmol) with Saponin added		
	NaCl histidine pH 7.6	149	21
	TRIS-HCl pH 7.6	48	25
	TRIS-maleate pH 5.8	170	22
III	ISOTONIC HEMOLYSIS		
	with saponia	181	not determined
	with ethylene glycol	10	"

DATA from Hanahan and Ekholm Arch Biochem Biophys 187 (1978) 170.

Enzyme activity = micromoles of Pi released per gram of hemoglobin per 2hr at $44^{\circ}C$.

Accumulation of calcium by sarcoplasmic reticulum in muscle is coupled to ATP hydrolysis by a membrane-bound (Ca^{2+} + Mg^{2+})ATPase. Hydrolysis of ATP and calcium translocation into the reticulum involves the transfer of γ-phosphate of ATP to the enzyme to form an acid-stable phosphorylated intermediate (Martonosi A. 1969). The formation of the intermediate requires Ca^{2+} and Mg^{2+} and its hydrolysis is activated by Mg^{2+} but inhibited by high concentrations of Ca^{2+} (Meissner 1973). Studies of the mechanisms of forward and backward reactions have suggested the existence of more than one phosphorylated intermediates in the ATPase reaction. Shigekawa and Dougherty (1978) isolated two phosphorylated enzyme intermediates in rabbit skeletal muscle and from their data they postulated the following reaction mechanism.

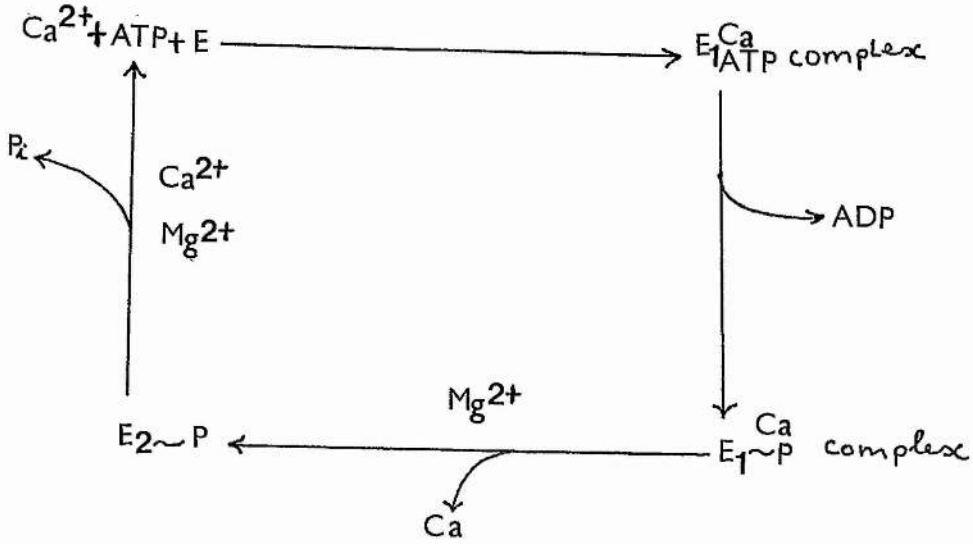


Figure 2 The (Ca^{2+} + Mg^{2+}) ATPase. E_1 and E_2 are the two phorylated enzyme intermediates.

The $(Ca^{2+} + Mg^{2+})$ATPase activities were investigated in sarcolemma in muscular dystrophy. Dhalla et al. (1973) reported elevated sarcolemma $(Ca^{2+} + Mg^{2+})$ATPase activity in Duchenne dystrophy. Investigations of $(Ca^{2+} + Mg^{2+})$ATPase of erythrocyte membranes indicated elevated activity in Duchenne muscular dystrophy and reduced activity in myotonic dystrophy (Hodson et al. 1977; Luthra et al. 1979). As with $(Na^{+} + K^{+})$ ATPase, activity of this enzyme also varied with method of cell lysis (Table II above).

Adenylate cyclase was another membrane-bound enzyme investigated. Mawatari et al. (1974) reported a lesser activation of the enzyme by fluoride and adrenaline in Duchenne muscle biopsies than in controls, but subsequent findings reported that the observation was not disease-specific. Subsequent studies by Mawatari et al. (1976) demonstrated that the basal activity of adenyl cyclase in cultured Duchenne myotubes was higher than controls and that the activity was not significantly stimulated by adrenaline and isoproterenol. This finding was reported to be disease-specific. The same authors reported that the basal adenyl cyclase activity in Duchenne erythrocyte membranes was twice that of controls and that adrenaline stimulated adenyl cyclase activity of normal membranes and had no effect on Duchenne erythrocyte membrane enzyme. Recently Tortolero et al (1979) have not been able to find any difference between the basal activities of the enzyme in freshly prepared erythrocyte ghosts from normal individuals and from Duchenne dystrophy patients.

4.5 Membrane lipids abnormalities

Phospholipids are a major component of cell membranes. They comprise

60% of the total lipids in membranes of most cell types. Alterations in the content of specific phospholipids have been described in the sarcoplasmic reticulum isolated from the muscle of patients with Duchenne muscular dystrophy. These include an increased proportion of sphingomyelin (Takagi et al., 1973) and decreased amount of phosphatidyl choline. In erythrocyte membranes from Duchenne muscular dystrophy patients, Kunze et al. (1973) and subsequently Kalofoulis et al., 1977 observed significantly elevated sphingomyelin and decreased levels of phosphatidyl ethanolamine and phosphatidyl serine. Evidence from Koski et al. (1978) and Kobayashi et al. (1978) failed to substantiate Kunze et al.'s findings. In contrast, they found no difference in erythrocyte membrane phospholipids between Duchenne patients and controls. This finding has recently been substantiated by McLaughlin and Engel (1979). Attention should perhaps be drawn here to the fact that the properties of membranes are fundamentally dependent on constituent phospholipids and cholesterol and that alterations of these in ratio or composition will profoundly affect cellular behaviour. The importance of membrane lipids in regulating the structures and the functions of membrane-bound enzymes can never be over-stressed.

4.6 Membrane Proteins

Since membrane proteins appear to be responsible for lipid bilayer stability receptor function and transport processes across the membrane, changes in these proteins may affect cellular function.

Earlier studies looked at the phosphorylation of endogenous membrane proteins in fresh erythrocyte ghosts prepared in the absence of Mg^{2+}. Studying protein bands II and III, defined as major peaks by electrophoresis on 6% SDS polyacrylamide gels, Roses et al. (1975) found an abnormally

elevated phosphorylation of these two bands in Duchenne muscular dystrophy erythrocyte ghosts. Decreased phosphorylation of band III was observed in myotonic dystrophy. Recently, Wong and Roses (1979) have fractionated band III glycoproteins into three fractions using affinity chromatography on Ricin communis-I sepharose 4B column. The fraction that eluted with 100 mM NaCl/100 mM D-galactose, comprising 3% of the total erythrocyte membrane proteins, was phosphorylated 50% less in membranes from myotonic dystrophy patients than in controls. Contrary to the above observation Iyer et al. (1977) and subsequently Tortolero et al. (1979) found no difference in the endogenous phosphorylation of spectrin and band III proteins in the membranes of Duchenne erythrocytes compared with the membranes from normal cells. It has recently been suggested that differing observations reported may be due to technical differences in methods of ghost preparation and age of erythrocytes.

Studies with electron spin resonance (e.s.r.) techniques have demonstrated reduced fluidity of erythrocyte membranes from Duchenne dystrophy patients (Wilkerson et al. 1978). It was suggested that this observation could be consistent with increased rigidity of membrane proteins in dystrophic erythrocytes.

Some investigations have looked at sarcoplasmic reticulum membrane proteins. Studies by Ikemoto et al. (1971) and McLennan (1975) have shown that several proteins could be isolated from sarcoplasmic reticulum including the following three major proteins:

(i) A 100 000 molecular weight protein which contains ATPase enzyme and calcium transport mechanism.

(ii) Calsequestrin (M.Wt. 55 000) which binds large quantities of calcium and

(iii) A 44 000 molecular weight protein which binds lesser quantities of calcium.

Investigations of sarcoplasmic reticulum membrane proteins from Duchenne dystrophy patients indicated a decrease in the amount of the 100 000 molecular weight protein and an increase in the 44 000 mol.wt. protein.

Polyacrylamide gel electrophoresis was also used in some investigations of membrane proteins. No reproducible differences in the protein patterns of erythrocyte ghosts from Duchenne patients and carriers, myotonic dystrophy patients and controls (Roses et al. 1975) could be seen. This finding was later substantiated by Kobayashi et al. (1978) and Tortolero et al. (1979). Despite these observations we have asked ourselves the question: "Are there really no differences in membrane proteins patterns between dystrophic and control erythrocytes or was the method used by these workers to analyse them not sensitive enough?" or, "Does each band in SDS gel represent a homogenous protein?" In this report we have attempted to answer these questions by employing a more sensitive two dimensional electrophoresis technique developed by O'Farrell (1975) and modified by Milkowski and Rubin (1978) to analyse erythrocyte membrane proteins from Duchenne dystrophy patients and carriers, limb-girdle muscular dystrophy patients and control subjects.

EXPERIMENTAL

5. MATERIALS AND METHODS

5.1 Theory to methods

Proteins are amphoteric substances i.e. each protein has a net charge that varies with the pH of its environment. This net charge represents the sum of positive and negative charges on the surface of a protein. When passing from a very low pH to a very high pH, the net charge changes in a continuous manner from plus to minus. At a well-defined pH the net charge equals zero. This point is called the iso-electric point (pI). Isoelectric focusing utilizes a stationary and stable pH gradient which increases towards the cathode. Such a gradient is brought about by using a mixture of specifically designed amphoteric substances called carrier ampholytes. If a protein is introduced at a point away from its pI, it will attain a net charge and under the influence of an electric field will move towards either of the electrodes depending on the sign of the net charge. As it moves, the net charge gradually decreases and finally when it reaches its pI, the net charge will equal zero and migration will cease. This phenomenon is used to separate proteins by concentrating them on their pI's and hence the term isoelectric focusing. The method can separate proteins having pI's differing by 0.01 pH units.

In SDS (sodium dodecyl sulphate) an oligomeric protein is dissociated into its subunits and completely denatured. The polypeptide chains bind a stoichiometric amount of negatively charged SDS on a weight basis and the resulting protein-SDS complexes approximate, in solution, to rigid rods (Fish et al., 1970; Reynolds, 1970). Proteins in SDS thus have the same charge-to-mass ratio (since the amount of SDS bound is sufficient to swamp out the intrinsic charge of all but exceptional proteins) and

the same shape. In an electric field the protein-SDS complexes all travel towards the anode and when electrophoresis is carried out in a polyacrylamide gel matrix, they are subject to molecular sieving and migrate according to their molecular weights. The small molecules travel fastest. In two dimensional gel electrophoresis (Isoelectric focusing/SDS gel electrophoresis) the two separations are done at right angles and give very good resolutions.

5.2 Introduction to methods

The prototype of all modern electrophoretic methods is the free boundary method first used by Tiselius (1937) to separate globulin into α, β and γ components. Since then a steady increase in resolution has taken place due to the introduction of filter paper (Kunkel, 1952), starch gels (Smithies, 1955) and acrylamide gels (Raymond et al., 1959) as supporting media. Further attempts to improve the electrophoretic separation led to the birth of isoelectric focusing (Svesson, 1961) and the introduction of stacking systems (Davis, 1964; Laemmli, 1970).

In 1968, Wrigley first reported a very good separation of serum proteins by combining isoelectric focusing and gradient gel electrophoresis. Since then a variety of two dimensional electrophoresis techniques have been introduced. Hayes and Wellner (1969) combined gel electrofocusing and electrophoresis, Maurer et al. (1972) combined cellulose acetate electrophoresis and isoelectric focusing and Barret et al. (1973) combined isoelectric focusing and SDS gel electrophoresis. The most adopted two dimensional procedure is the isoelectric focusing-SDS gel electrophoresis method developed by O'Farrell (1975). This technique is a powerful tool for the resolution of complex mixture of proteins. It separates proteins on the basis of two independent very high resolving parameters, isoelectric point and molecular weight. Whilst isoelectric

focusing can discriminate peptides with a charge difference of 0.01 pH units, SDS gel electrophoresis can clearly separate molecules differing in molecular weight by 1 000 daltons. The technique can separate proteins with the same net charge but different molecular sizes and also those with different charges but similar molecular sizes. Many laboratories have adopted this method to detect some products of many gene loci. We, here, have adopted the method to detect some gene products in genetic muscular dystrophies.

5.3 Materials

(a) Lysis buffer

- 9.5M urea
- 2% (w/v) NP-40
- 2% (w/v) ampholines
  - 1.6% pH 5-7
  - 0.4% pH 3.5-10
- 5% (v/v) 2-mercaptoethanol

(b) 30% stock acrylamide for isoelectric focusing

- 2.84g acrylamide
- 0.16g methylene bisacrylamide
- q.s. 10ml with distilled water

(c) Stock nonidet P-40

- 10% (w/v) NP-40 in distilled water

(d) Sample overlay solution

6M urea

0.4ml pH 5-7 ampholines

0.1ml pH 3.5-10 ampholines

q.s. 25ml with distilled water

(e) Catholyte

0.02M sodium hydroxide

(f) Anolyte

0.01M orthophosphoric acid

(g) Separation gel buffer (1.5M Tris-HCl, pH 8.8)

18.3g Tris base

50ml distilled water

Adjust to pH 8.8 with 1M HCl

q.s. 100ml with distilled water

(h) Stacking gel buffer (0.5M Tris-HCl pH 6.8)

6.0g Tris base

50ml distilled water

Adjust to pH 6.8 with 1M HCl

q.s. 100ml with distilled water

(i) 30% stock acrylamide for SDS separation gels

30g acrylamide

0.15g N'N' methylene bisacrylamide

q.s. 100ml with distilled water

(j) 10% stock acrylamide for stacking gel

10g acrylamide

0.15g NN' methylene bisacrylamide

q.s. 100ml with distilled water

(k) SDS sample buffer

10% (w/v) glycerol

5% (w/v) 2-mercaptoethanol

2.3% (w/v) sodium dodecyl sulphate

0.0625M Tris-HCl pH 6.8

(l) Electrode buffer

30.3g Tris base

144.0g glycine

5.0g SDS

q.s. 5l with distilled water

(m) Stock grinding solution pH 7.4

9.5M urea

1% (w/v) SDS

10% (v/v) 2 mercaptoethanol

(n) Lysis supplement

4ml 10% (w/v) NP-40

0.08ml ampholines pH 3.5-10

0.32ml ampholines pH 5-7

(o) Fixing solution (15% TCA)

15g Trichloroacetic acid

q.s. 100ml with distilled water

(p) Staining solution

1.25g Coomassie blue R250

227ml methanol

227ml water

46ml glacial acetic acid

(q) Destaining solution

250ml methanol

75ml glacial acetic acid

675ml water

(r) Bromophenol blue (Tracking dye)

0.01% (w/v) Bromophoneol blue

(s) 10mmol Tris-HCl pH 8.0

1.21g Tris base

Add 500ml water

Adjust to pH8 with 1M HCl

q.s. 1l with distilled water

(t) 0.15M sodium chloride

8.7g sodium chloride

Add 500ml distilled water

Adjust to pH 8 with 5mM phosphate buffer

q.s. 1000ml with distilled water

(v) 0.1M stock $Na_2HPO_4 \cdot 12H_2O$

35.8g disodium hydrogen orthophosphate

q.s. 1000ml with distilled water

(w) 0.1M stock $NaH_2PO_4 \cdot 2H_2O$

15.6g sodium dihydrogen orthophosphate

q.s. 1000ml with distilled water

(x) 56mM sodium borate buffer pH 8.0

3.46g boric acid

Add 500ml distilled water

Adjust to pH 8.0 with 1M NaOH

q.s. 1000ml with distilled water

5.4 Methods

5.4.1 Preparation of red cell membranes

Red blood cell membranes were prepared at 4°C according to Rubin and Milikowski (1978). About 20ml of whole citrated blood were spun at 5 000g for 10 minutes and the serum and the buffy coat were aspirated off. The red blood cells were then washed three times in phosphate-buffered saline (0.15M saline buffered to pH8 with 5mM sodium phosphate). At each washing, the sample was spun at 5 000g and the buffy coat and top layer of cells were removed. The cells were then lysed twice in 5mM sodium phosphate buffer pH8 and the membranes were spun at 10 000g for 10 minutes. The membranes were then resuspended in 10mM Tris-HCl buffer, pH8, and then spun at 10 000g for 10 minutes. The membranes were then washed twice in 56mM sodium borate buffer pH8.0 and spun at 10 000g for 10 minutes the first wash and at 50 000g for 10 minutes the second time (Figure 3).

5.4.2 Solubilisation of Red cell membranes

This was done essentially according to Rubin et al. (1978)'s modification of Wilson et al's method (1977a). 90µl of stock grinding solution were added to the membranes prepared as shown above. The mixture was then shaken for 30 seconds at room temperature. 30µl of lysis supplement were then added and the sample shaken for a further

30 seconds. Finally 120μl of lysis buffer were added. After shaking, the sample was ready for application onto an isoelectric focusing gel.

5.4.3 Isoelectric focusing

This was done according to the method of O'Farrel (1975). The gel mixture consisting of

5.5g urea

1.3ml of 30% acrylamide solution (b)

2.0ml water

2.0ml of 10% (w/v) NP-40

0.4ml ampholines pH5-7

0.1ml ampholines pH3.5-10

was made in a side arm flask. The flask was swirled with warming to dissolve the urea and then degassed for a minute. Polymerisation was achieved by adding 10μl of TEMED and 10μl of 10% (w/v) ammonium persulphate. The mixture was loaded into glass tubes 13cm long by 2mm inside diameter. The tubes were filled to 12.5cm mark and then overlaid with 20μl of sample overlay solution (d) and 20μl of water. The gel was left to set at 37^{o}C for 2 hours. After setting, the tubes were assembled in a standard electrophoresis chamber as described by O'Farrell and pre-run at

(i) 200 volts for 15 minutes

(ii) 300 volts for 30 minutes and

(iii) 400 volts for 1 hour.

The samples were then loaded, overlayed with sample overlay (d) and then run at room temperature at a constant voltage of 400 volts for 18 hours. After the run, the gels were removed from the tubes. Some of the gels were used for pH gradient determination and the rest were equilibrated

in SDS-sample buffer (k) for at least 30 minutes and then run into SDS gel in the second dimension.

5.4.4 pH gradient determination

The gel for pH gradient determination was cut transversely into 1cm pieces. Each of these pieces was placed in a tube containing 1ml of 30mM potassium chloride. After standing for about 16 hours, the pH value in each tube was measured by a pH meter at 20°C. The pH gradient obtained is shown in results.

5.4.5 Second dimension SDS gel electrophoresis

The second dimension gel electrophoresis employed the SDS buffer systems first described by Laemmli (1970). The slab gels were made in double glass plates almost similar to those described by O'Farrell (1975). Perspex strips 1mm thick were used as spacers and a little vaseline was used to make a water-tight seal. Separation gels of uniform concentrations were prepared from the recipes shown in Table III below. The volume in each case was enough for 2 plates (i.e. 2 separation gels).

TABLE III

GEL STRENGTH %	VOL OF 30% ACRYL SOLUTION (i)(ml)	VOL OF 10% SDS (ml)	VOL OF SEPARATION BUFFER (G) (ml)	VOL OF WATER (ml)		VOL OF TEMED (µl)	VOL OF APS (ml)
6	10	0.5	6.25	33	DEGAS	10	0.25
9	15	0.5	6.25	28		10	0.25
12	20	0.5	6.25	23		10	0.25
15	25	0.5	6.25	18		10	0.25
18	30	0.5	6.25	13		10	0.25
21	35	0.5	6.25	8		10	0.25
24	40	0.5	6.25	3		10	0.25

The 9% separation gel we mostly used was made my mixing

15ml of 30% acrylamide solution (i)

0.5ml of 10% (w/v) SDS

6.25 separation buffer (g) and

28.0ml water..

The mixture was degassed for 2 minutes before adding 10µl of TEMED and 0.25ml of 10% (w/v) ammonium persulphate. The mixture was poured into the mould to a level 20mm below the bottom of the norch. The mixture was overlayed with about 1ml of water. The mixture was left to polymerise for 2 hours, after which the water was removed and then 5% stacking gel added to fill the gel mould. Five per cent stacking gel was prepared by mixing

10ml of 10% acrylamide solution (j)

0.2ml of 10% (w/v) SDS

4.8ml of stacking buffer (h) and

4.8ml of water.

The mixture was degassed and then 10μl of TEMED and 0.2ml of 10% (w/v) ammonium persulphate were added to achieve polymerisation. The mixture was overlayed with a pasteur pipetteful of water and left to polymerise for 1 hour. The first dimension gel (isoelectric focusing gel) was then loaded into the SDS gel and sealed with 1% (w/v) agarose, mixed with 0.01%(w/v) bromophenol blue made in sample buffer (k). The lower tank of the electrophoresis apparatus was filled with electrode buffer (L), the plates loaded and then the top tank was filled with the same buffer. Electrophoresis was run at 4mA per plate until the tracking dye (bromophenol blue) was about 5mm from the bottom of the gel.

5.4.6 Detection of peptides

After the run, the gel was removed from the plates and fixed with 15% (w/v) trichloroacetic acid (solution O) for 30 minutes. The gel was then stained for 2 hours with Coomassie brilliant blue R250 solution (P). The gel was finally destained by soaking in several changes of destaining solution (g) and the protein pattern obtained photographed by a 35mm camera.

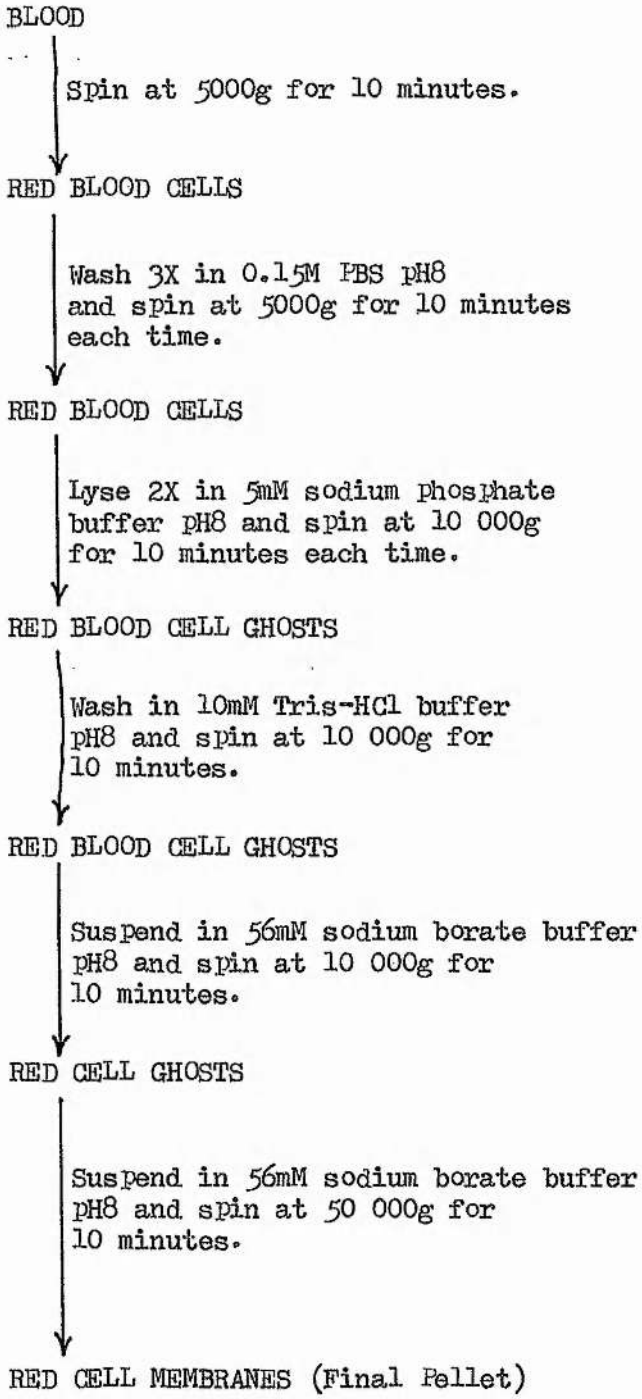


FIGURE 3

Flow diagram for preparation of red cell membranes.

RESULTS

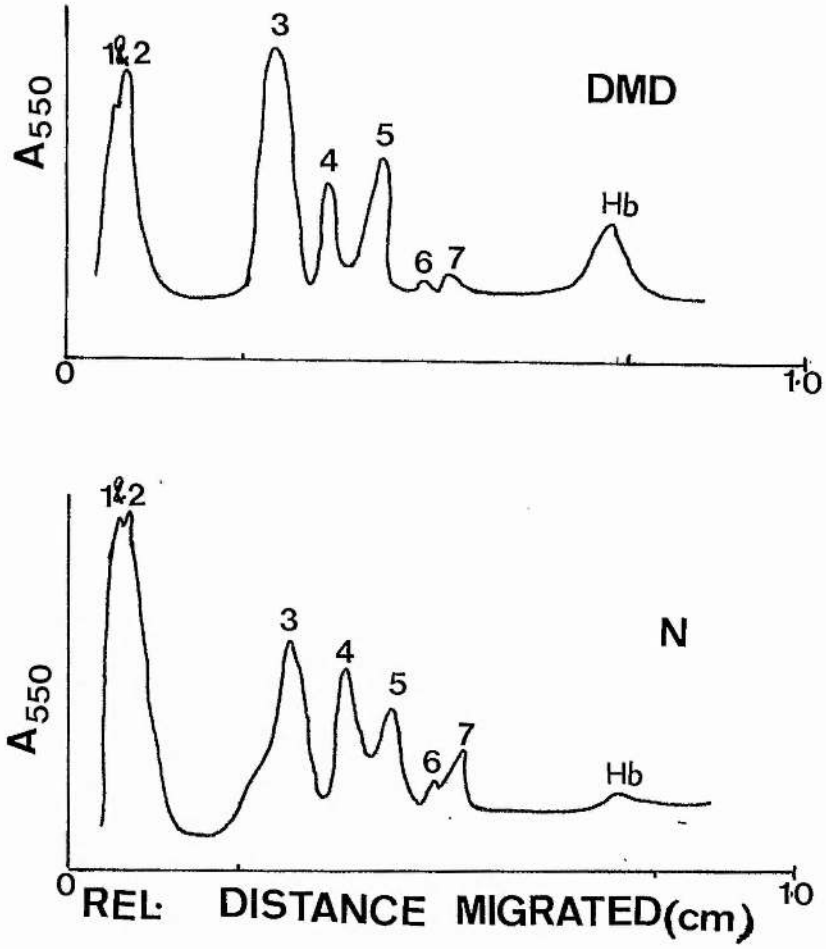


Figure 4 Densitometry recordings of Coomassie blue R250 stained SDS-polyacrylamide gel electrophoretic polypeptide pattern of erythrocyte membranes from Duchenne Dystrophy patients (DMD) and normal control (N). The polypeptides were separated in 9% gels.

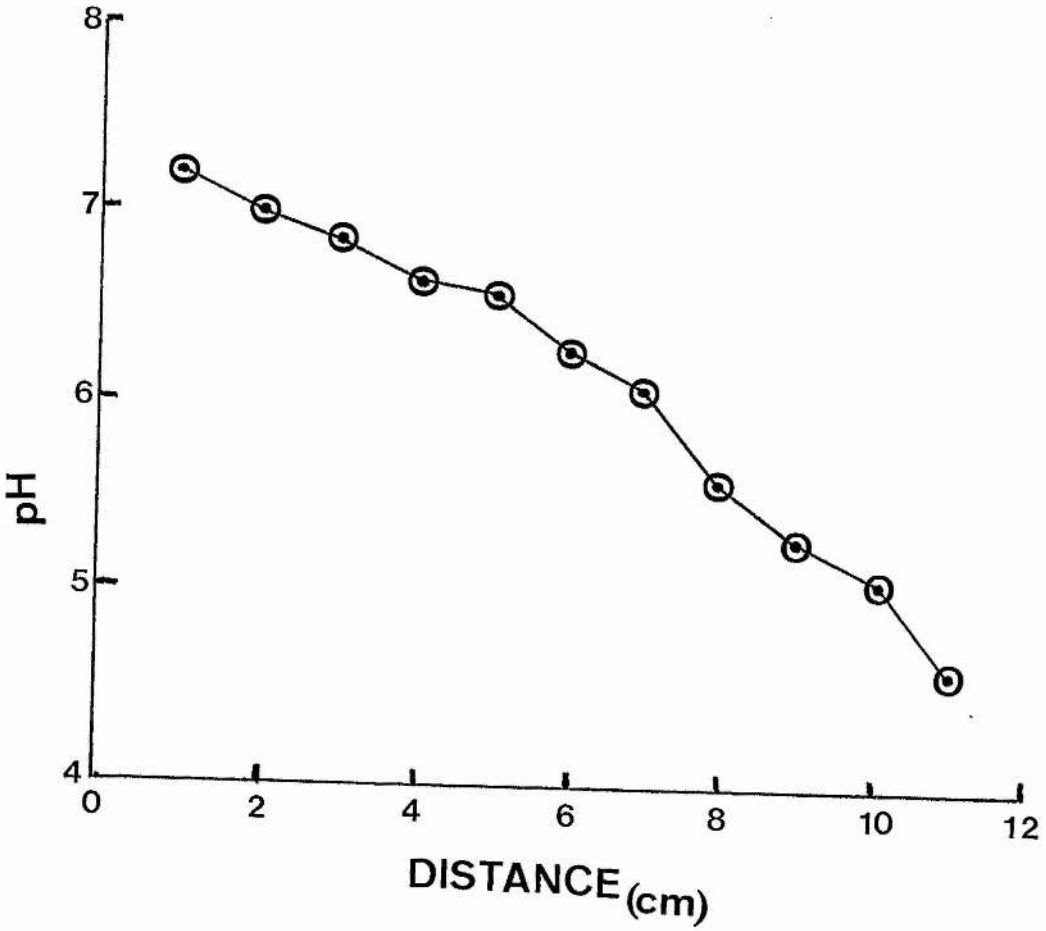


Figure 5 pH gradient for isoelectric focusing gel (first dimension gel). The zero point on the abscissa is the negative end of the gel i.e. origin of electrofocusing.

RESULTS

6.2 Solubilisation of erythrocyte membranes

Erythrocyte membranes were prepared by lysing cells in hypotonic solution buffered at pH 8. The washed membranes were then solubilised in stock grinding solution/lysis supplement/lysis buffer. This procedure completely solubilised the membranes because spinning of the solubilised membranes at 50 000g for 30 minutes failed to produce any pellet. The solubilised membranes proteins were run on two dimensional gel as described in methods. The isoelectric focusing gel consisted of 4% acrylamide with a cross-linking of 5%. The pH gradient was formed by 2% ampholytes comprised of 0.1ml pH 3.5 - 10 and 0.4ml pH 5 - 7 ampholines. After focusing at 350 volts, constant voltage, for 18 hours, the pH gradient for the isoelectric focusing gel, figure 5, was obtained by

i) cutting the gel into 1cm pieces

ii) washing the slices overnight in 1ml aliquots of 30mM KCl and

iii) measuring pH values of 1ml KCl aliquots at 20°C.

The pH gradient curve obtained lay between pH 4.7 and pH 7.2 (Figure 5).

The samples run on single dimension SDS-PAGE (Figure 4) were first dissolved in SDS sample buffer before loading onto 9% acrylamide acrylamide gels. Figure 4 shows the typical scan patterns obtained for the normal control and Duchenne muscular dystrophy samples. No consistent differences were observed between Duchenne muscular dystrophy samples and those from normal controls.

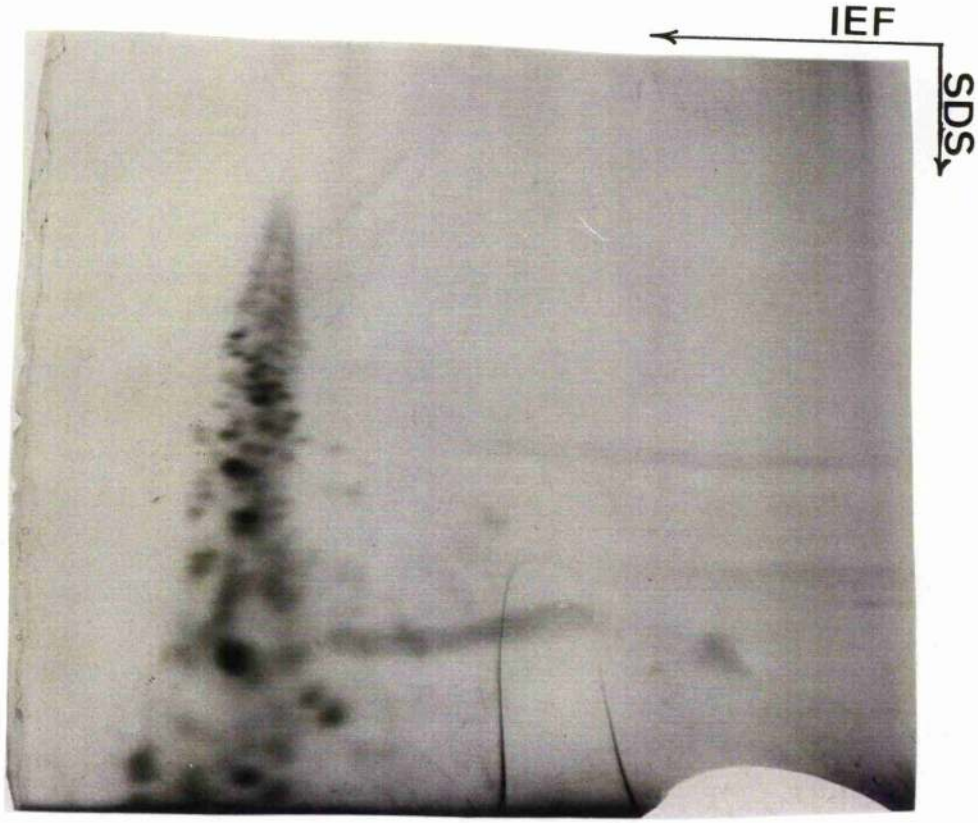


Figure 6 A pattern of protein spots from a preparation of erythrocyte membranes from a normal control subject. 0.8mg of proteins were applied on a first dimension gel. The two dimensional gel is shown with acid end to the left and SDS electrophoresis proceeding downwards in 9% gel.

Figure 7 Two dimensional gel protein patterns from a normal control subject. 0.7mg of erythrocyte membrane proteins were separated in 12% gel in the second dimension.

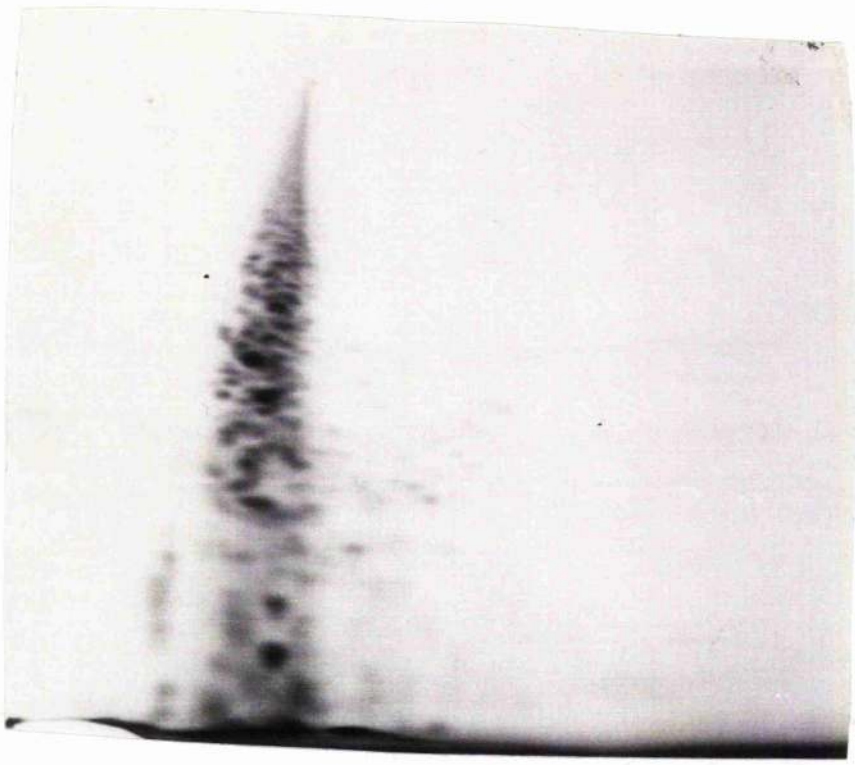


Figure 8 Protein patterns of erythrocyte membranes from Duchenne muscular dystrophy carrier. 0.8mg of proteins were separated in 9% gel, in the second dimension.

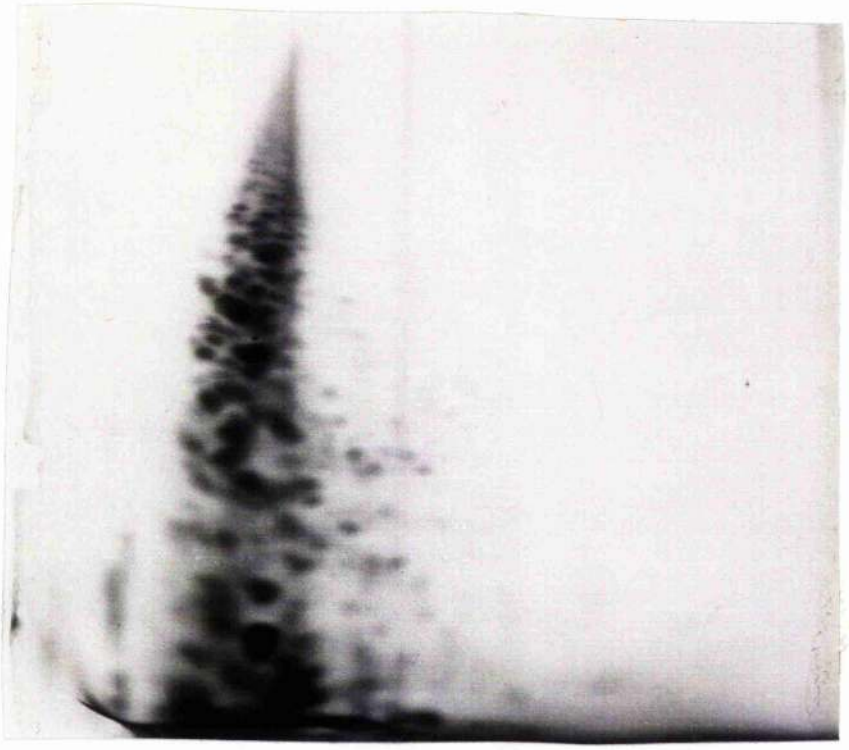


Figure 9 Same as Figure 8 except that 1.2mg of proteins were separated.

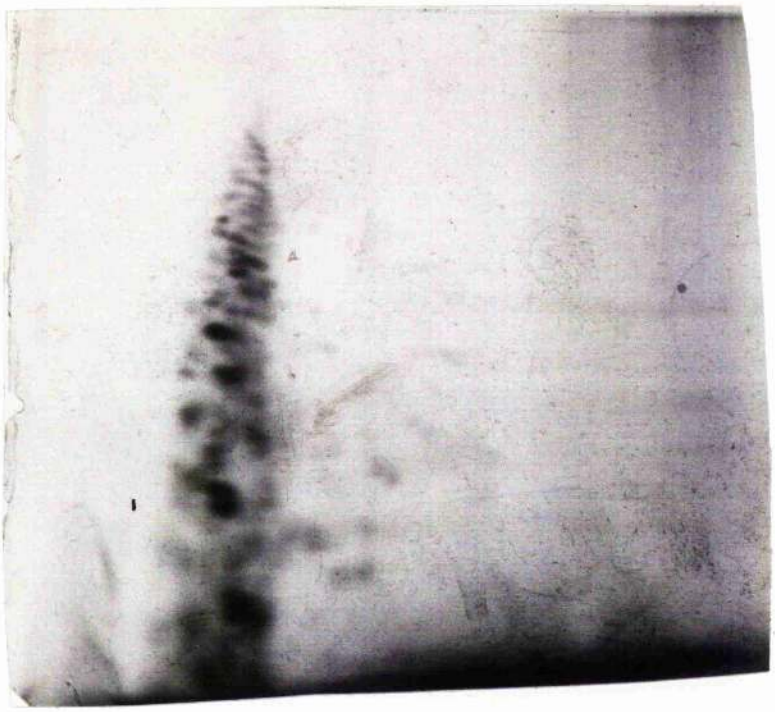


Figure 10 Protein patterns for erythrocyte membranes from Duchenne muscular dystrophy patient. 0.8mg of proteins were separated in 9% gel in the second dimension.

6.3 Two dimensional gel electrophoresis

The quantities of protein loaded onto the isoelectric focusing gels were determined by the method of Lowry et al. (1951). The isoelectric focusing procedure was carried out according to the method of O'Farrell (1975) as described in methods. The 9.5M urea/2% NP-40 in the isoelectric focusing gels maintained the proteins in a denatured state. After the run the isoelectric focusing gels were run at right angles into 9%, 12% or 15% discontinuous SDS gels. Figures 6 and 7 show the typical results obtained by running in 9% and 12% SDS gels respectively. The results suggest that the commonly described one dimensional sodium dodecyl sulphate protein of human erythrocyte membranes may represent an oversimplification of the numbers of individual polypeptides present within this structure. About one hundred and fifty peptides were visualised in 9% gels when stained with Coomassie blue R250. The 9% gels were able to clearly resolve proteins of sizes larger than 20 000 daltons. Figures 8, 9 and 10 show separations in 9% gels of samples from Duchenne muscular dystrophy patients and carriers. When o.8mg of proteins were loaded to the first dimension gels (Figures 6, 8 and 10), only the more abundant peptides were clearly visualised. The visualisation of minor components required massive overloading of the first dimension gels. In Figure 9 1.2mg of proteins were analysed. Some minor components became apparent and about 200 peptides could be counted in Figure 9. The use of 7-24 per cent gradient gels resolved over 300 peptides (Figure 19).

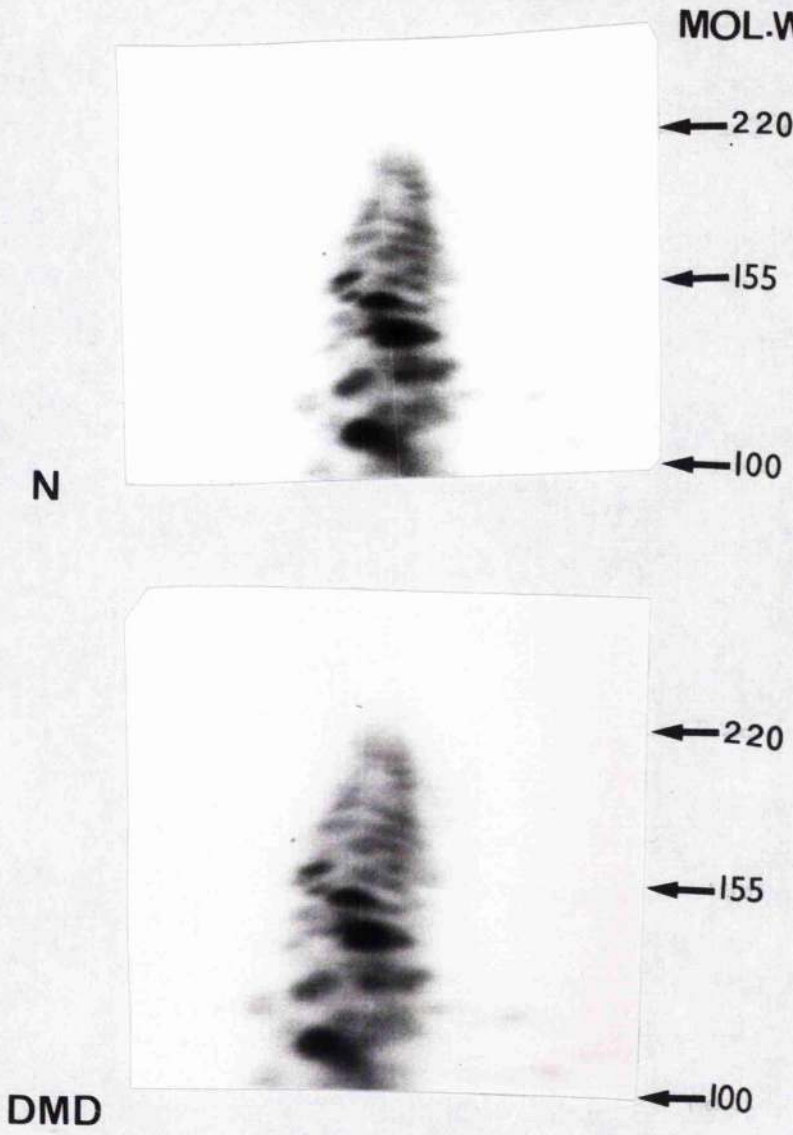


Figure 11 Sections from 12% gels showing the high molecular weight erythrocyte membrane protein patterns of Duchenne muscular dystrophy patients and normal control subjects.

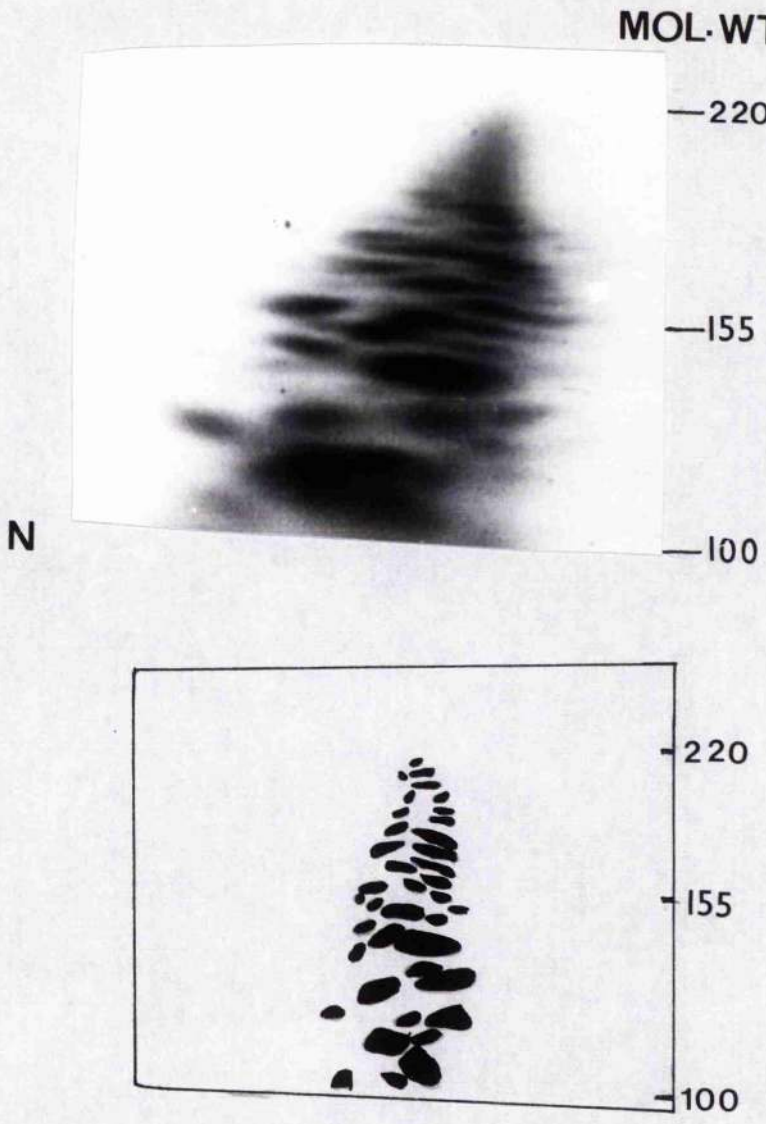


Figure 12 Section from 15% gel showing the high molecular weight protein patterns of normal control subject. Mapping of peptides in Figure 11.

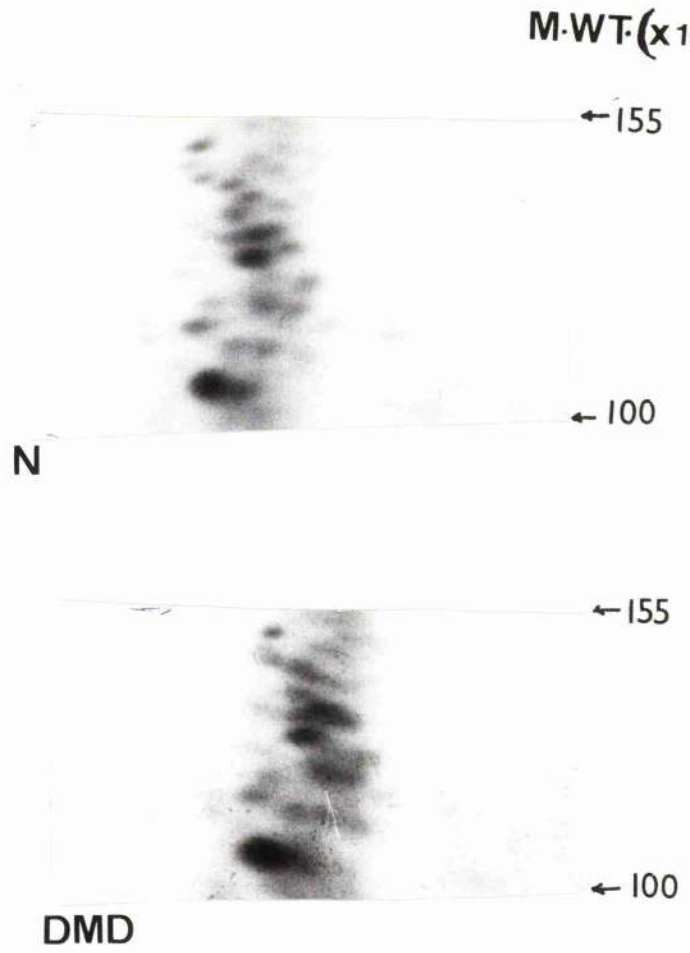


Figure 13 Sections from 9% gels including peptides of molecular weights 100 000 - 155 000.

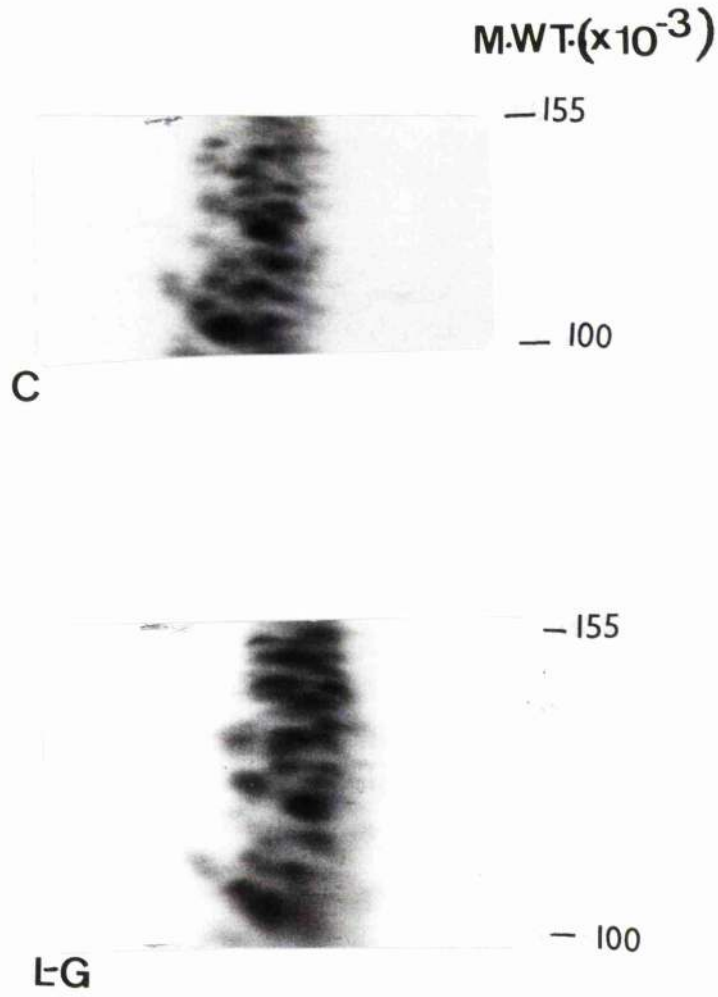


Figure 14 Sections from 9% gels as in Figure 13, but the peptides were of erythrocyte membranes from Duchenne muscular dystrophy carriers (C) and Limb-girdle dystrophy patients (LG).

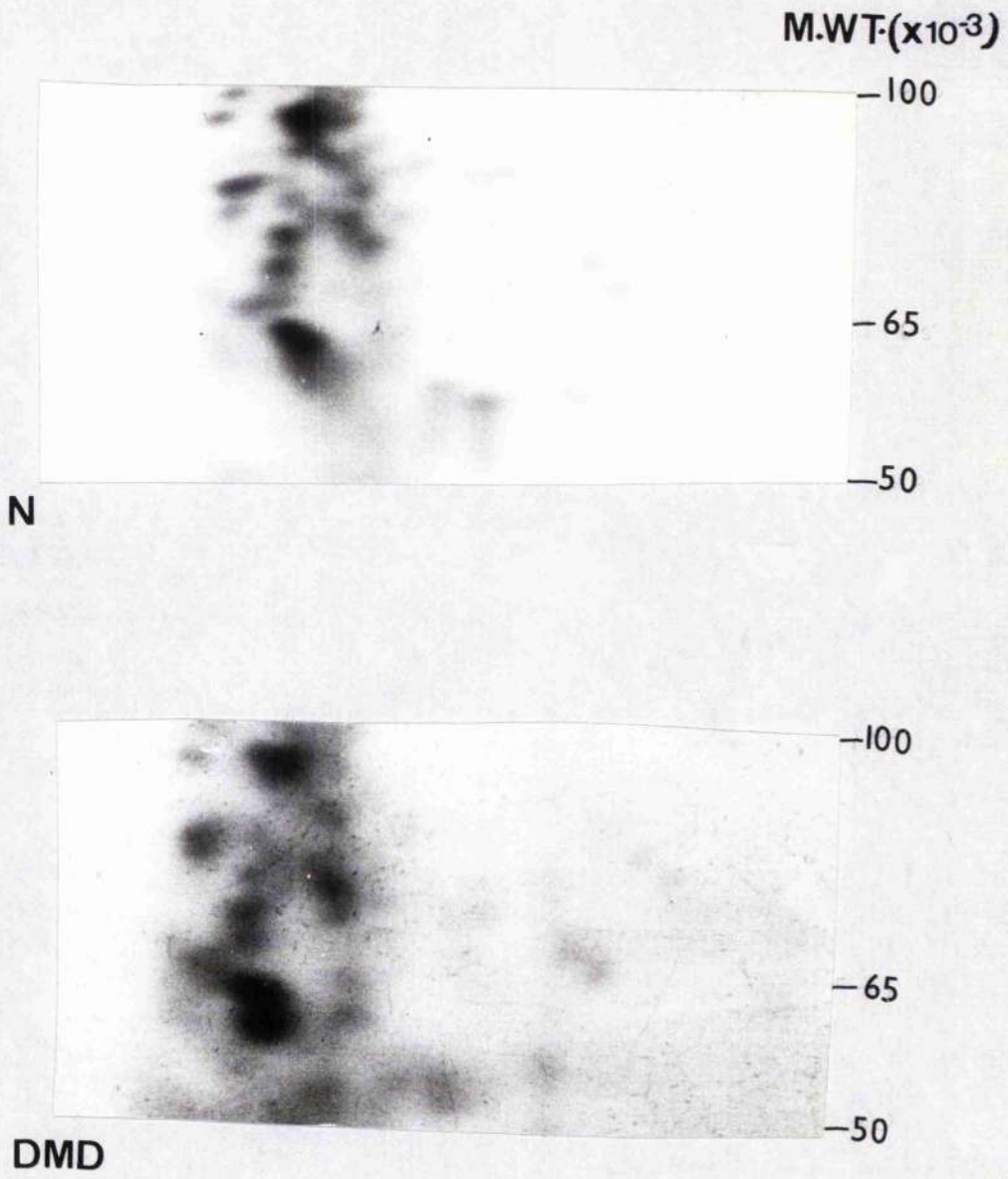


Figure 15 Sections from 9% gels showing the low molecular weight erythrocyte membranes peptide patterns.
DMD-Duchenne muscular dystrophy patients
N - normal controls.

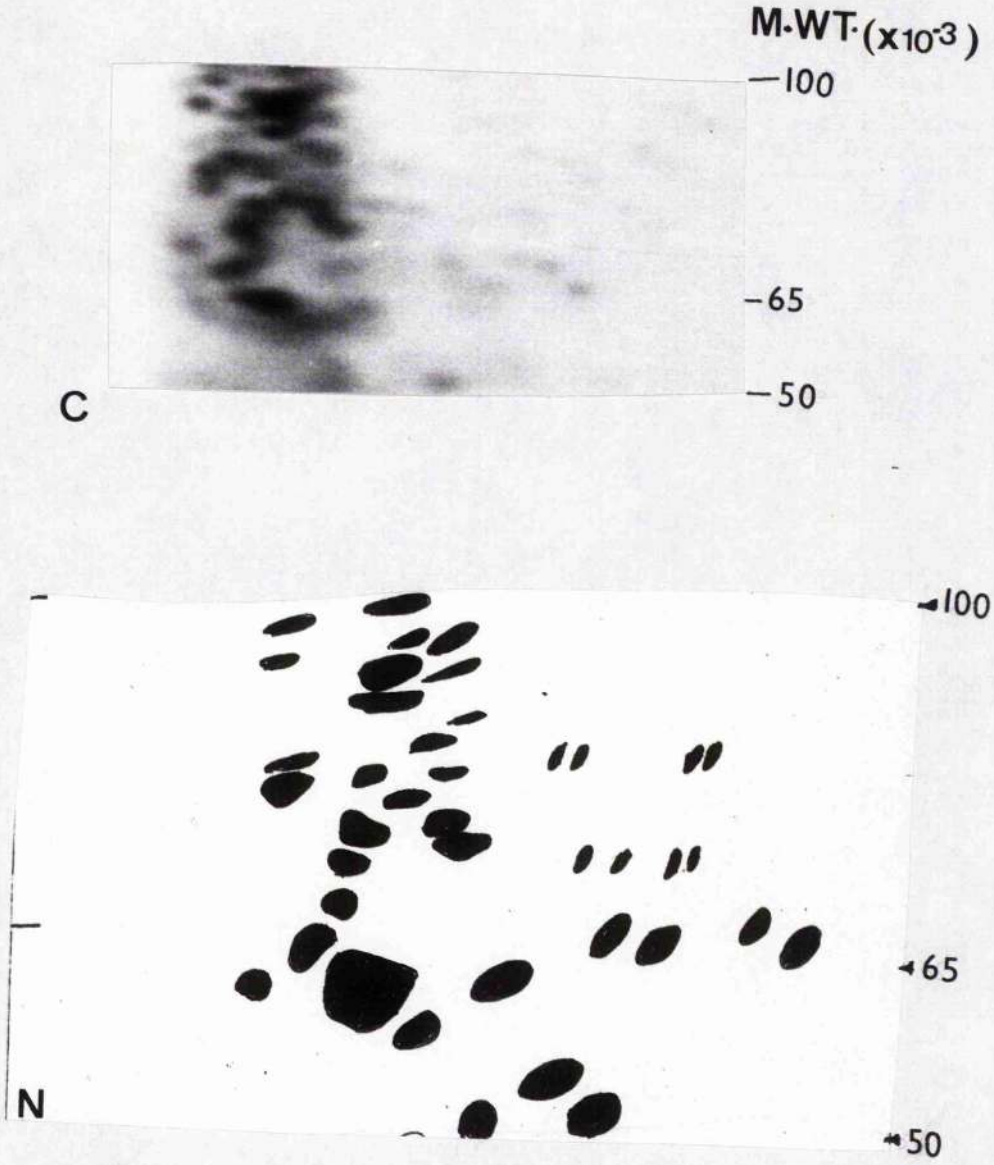


Figure 16 Section from 9% gel showing low molecular weight erythrocyte membranes peptide patterns. The membranes were prepared from Duchenne muscular dystrophy carrier (C). Lower part shows the map of the patterns from normal control samples (N).

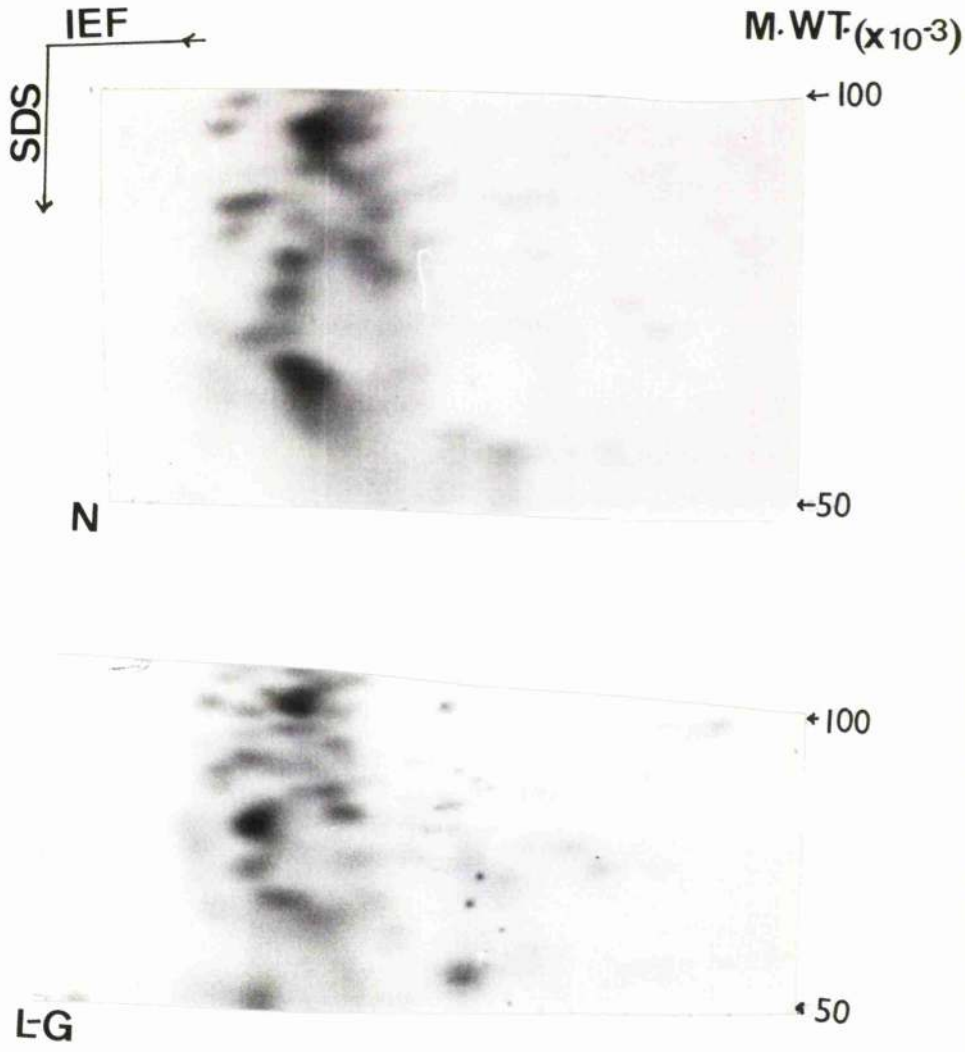


Figure 17 As in Figure 15 except that the patterns were of samples from limb-girdle (L-G) dystrophy patients and normal control subjects.

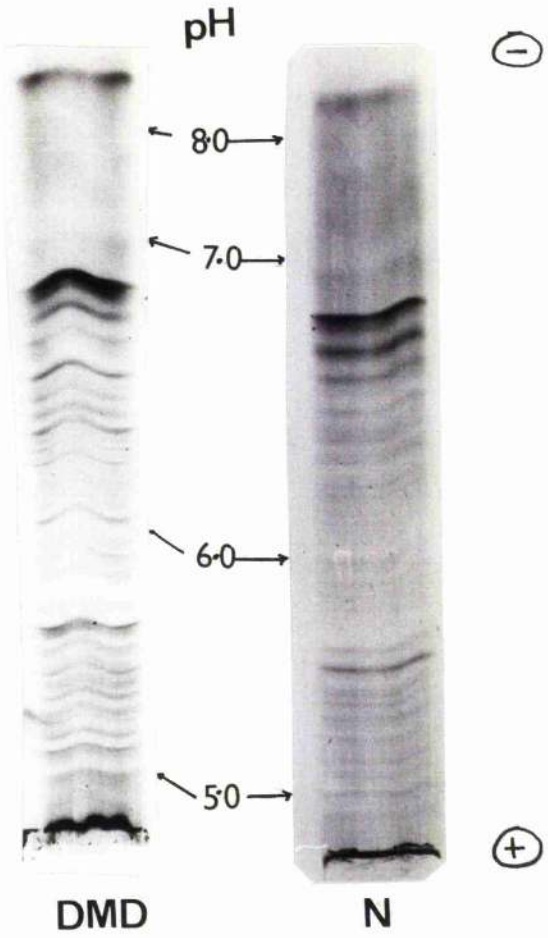


Figure 18 Electrofocusing of denatured erythrocyte membrane proteins from Duchenne muscular dystrophy patients and control subjects on a thin layer Ampholine polyacrylamide gel plate pH 3.5 - 10.

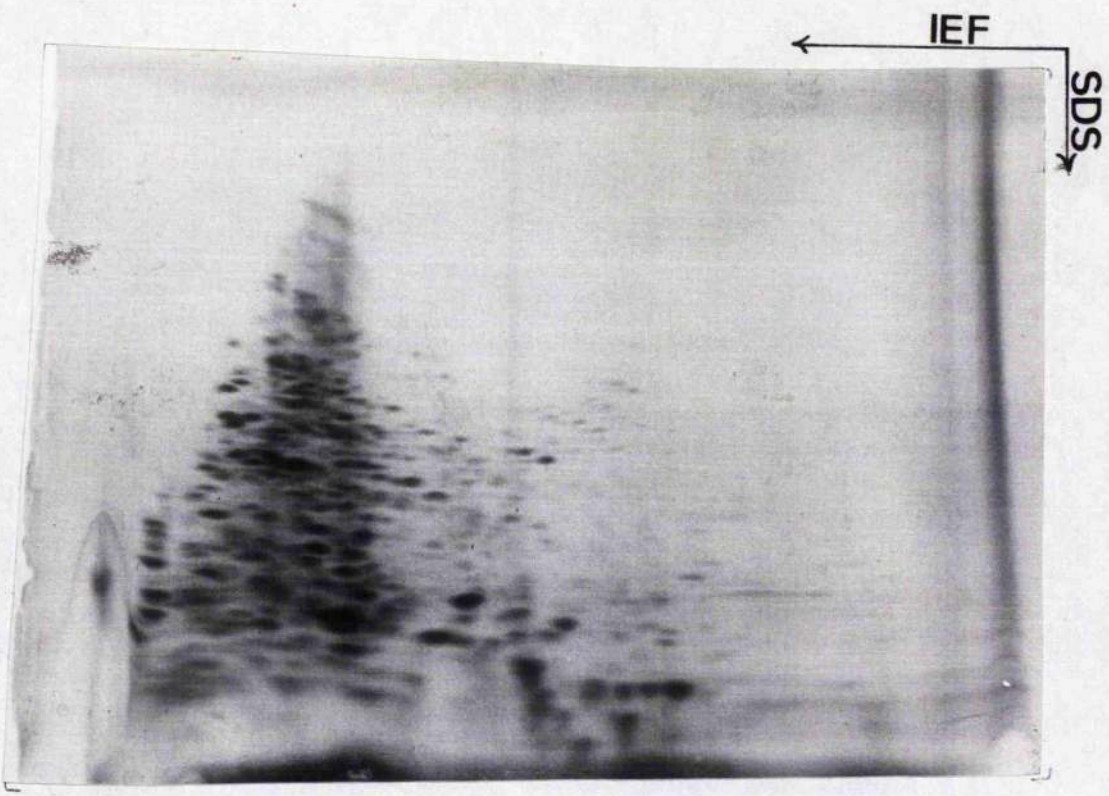


Figure 19 Protein patterns of erythrocyte membranes from normal control subject separated on 7-24% linear gradient gel, in the second dimension. 1.2mg of protein was applied on the first dimension gel.

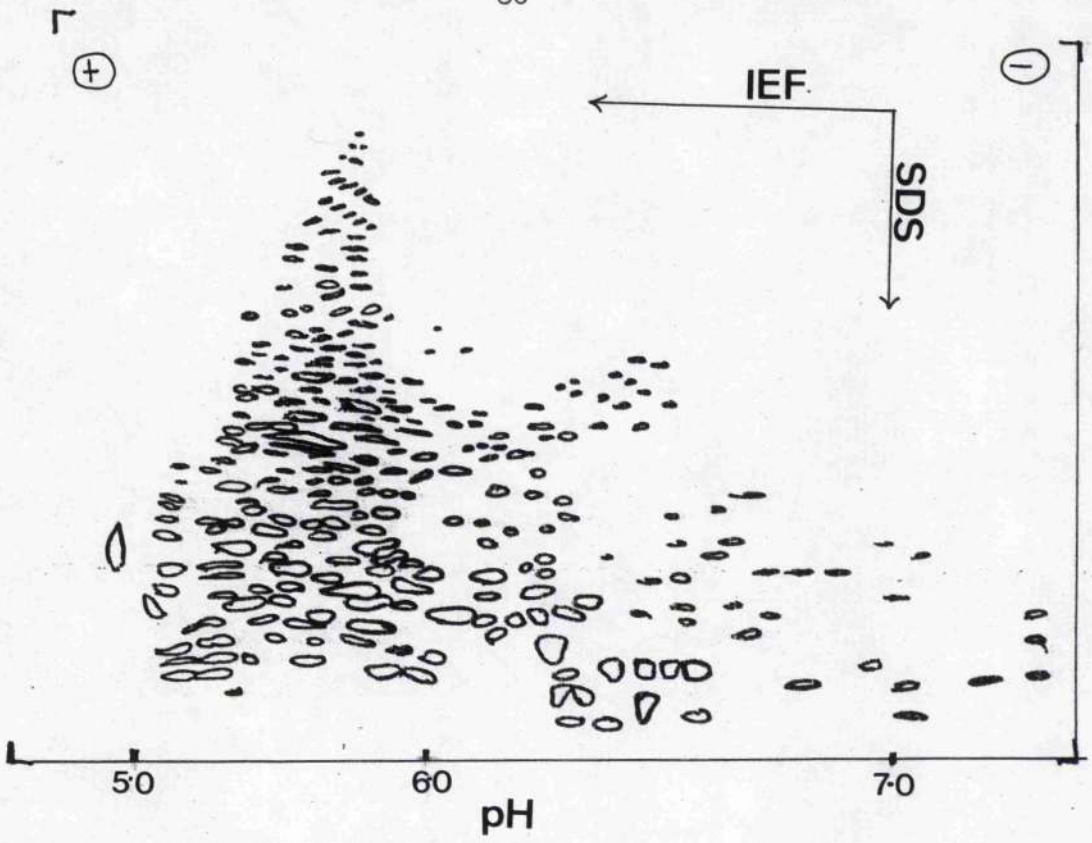


Figure 19b Diagrammatic representation of the same gel shown in Figure 19 with the pH axis and the directions of isoelectric focusing (IEF) and SDS gel electrophoresis (SDS) shown.

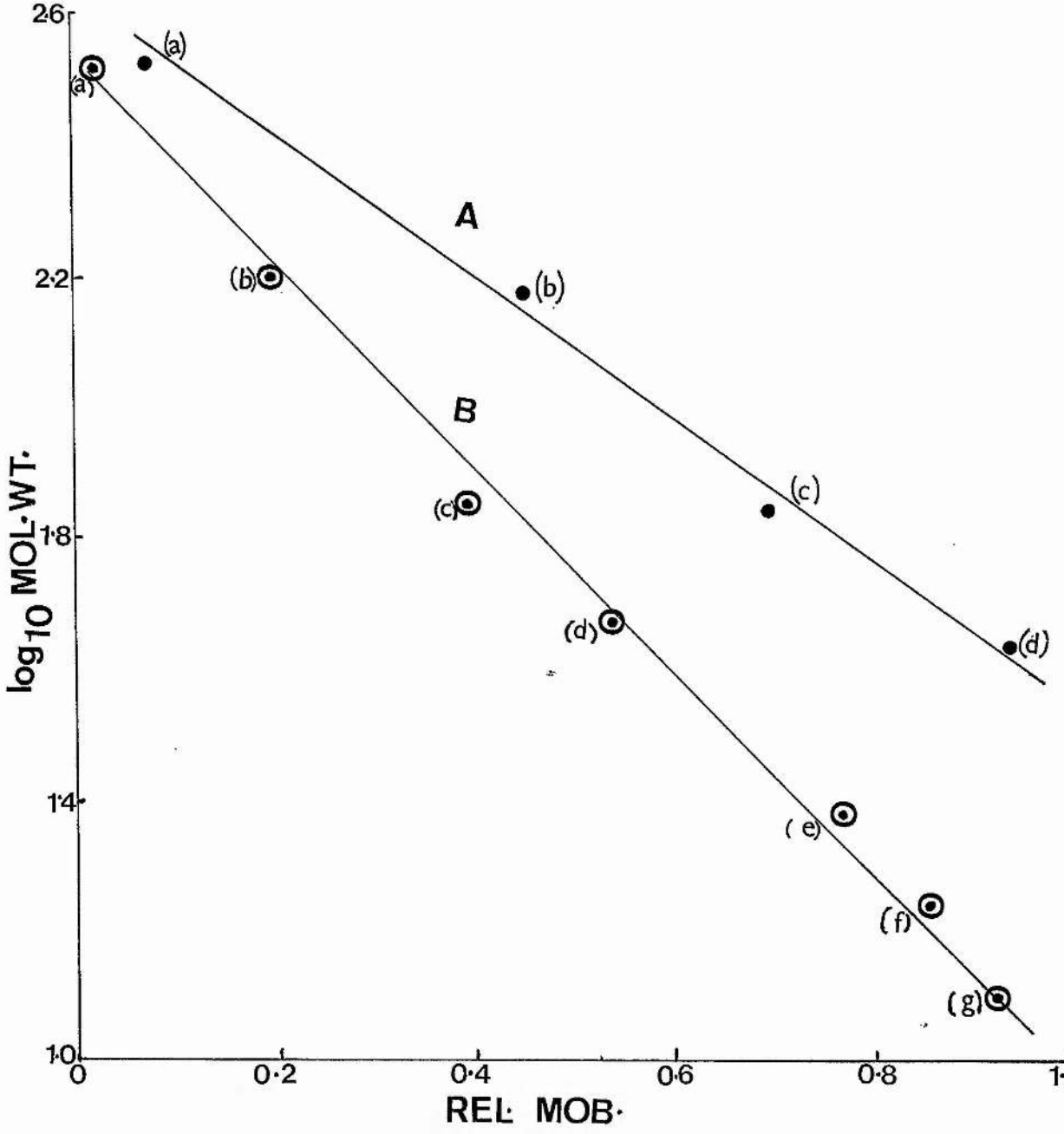


Figure 20 Mobility curves of standard proteins in (A) 9% gel and (B) 15% gels. Samples:
(a) fibrinogen (b) γ-globulin
(c) bovine serum albumin (d) ovalbumin
(e) trypsin (f) myoglobin (g) cytochrome C

6.4 Searching for variants

Figures 11-17 show sections of two dimensional gels illustrating methods of comparing samples from normal control subjects, Duchenne muscular dystrophy carriers and patients and limb-girdle dystrophy patients. Polymorphisms were not evident from comparisons of peptides larger, in apparent molecular weight, than 50 000. Since prolonged storage at -80°C is known to produce pronounced changes in protein patterns (Rubin and Milikowski (1978)), polymorphisms observed for peptides smaller than 50 000 daltons might have been due to differences in lengths and conditions of storage of samples at centres that provided us with test samples.

6.5 Isoelectric focusing of completely denatured erythrocyte membrane proteins in thin layer polyacrylamide gels.

The most common uses of analytical electrofocusing in thin layer polyacrylamide gels include:

(I) checking purity of samples

(II) Determining isoelectric points

and(III) Analysis of protein heterogeneity.

The technique is of great importance because of (a) reproducibility and (b) excellent resolving power. Here we used analytical isoelectric focusing in an attempt to type genetic polymorphism in Duchenne muscular dystrophy.

The thin layer polyacrylamide analytical isoelectric focusing was performed on LKB multiphor in pH 3.5 - 10 gradient. The erythrocyte membrane proteins analysed were completely denatured by solubilising them in stock grinding solution/lysis supplement/lysis buffer at 20°C.

Figure 18 shows the erythrocyte membrane protein patterns obtained

for Duchenne muscular dystrophy and, normal control samples. No genetic variants were seen by this method. The isoelectric focussing method was adopted for typing variants in Duchenne muscular dystrophy because of its success in typing Haemoglobin and alpha-1-antitrypsin variants (Jeppsson, 1977).

7.1 Discussions

For any human genetic disease, some protein must either be lacking or be so altered by mutation that its function is defective and thus causes the symptoms of the disease. The altered protein may result in abnormal composition and altered function of surface membranes. The abnormality in plasmalemma could involve a structural protein or an enzyme of the membrane or it could be that an abnormal enzyme has led to an abnormal placement of lipids, proteins or glycoproteins.

Current evidence suggests that the functional genetic fault of muscular dystrophies affects an enzyme or a structural protein which is decreased in amount or rendered functionally abnormal because of an altered amino acid sequence. In either case, the altered protein results in abnormal composition and altered function of surface membranes of muscles and other cells particularly erythrocytes. Erythrocytes in Duchenne muscular dystrophy have been reported to have alterations in K^+ (Shaafi et al., 1975) and Ca^{2+} (Plisher et al., 1978) permeability, increased osmotic fragility (Somers et al., 1979; Kim et al., 1980), increased electrophoretic mobility (Bosman et al, 1976), elevated ($Ca^{2+} + Mg^{2+}$) ATPase (Luthra et al., 1979), reduced oubain inhibition of ($Na^+ + K^+$) ATPase (Pearson T., 1978) and abnormal adenine metabolism (Solomons et al., 1977). Of the reported abnormalities in red blood cells, there is controversy of about 60 per cent of the observations and others await confirmation. Despite these difficulties, it is generally agreed that erythrocyte membranes are defective in muscular dystrophy disorders. The physical state of plasma membrane, which is determined by the type of phospholipids, the chain length of fatty acids, the relative proportion of saturated and unsaturated

fatty acids and the cholesterol content and the state of interactions of lipids and proteins, influences the activities of membrane-bound enzymes and the permeability of membranes for ions and other substances. Therefore, an alteration of a lipid or protein composition of erythrocyte membranes in muscular dystrophies may be responsible for the observed structural and functional changes.

In the present study, SDS polyacrylamide gel electrophoresis was performed on erythrocyte membrane proteins obtained from normal control subjects and Duchenne muscular dystrophy patients. Similar protein patterns were obtained as shown in Figure 4. The results obtained here agree with those previously reported by Roses et al. (1976), Kobayashi et al. (1978) and Tortolero et al. (1979). Sodium dodecyl sulphate polyacrylamide gel electrophoresis of red blood cell membrane proteins according to Fairbanks et al. (1971) produced, as expected, seven protein bands, numbered 1 to 7 in Figure 4. There is no prior reason to assume, however, that any particular stainable band is homogenous and indeed two dimensional gel electrophoresis of red blood cell membrane proteins produced over two hundred peptides (Rubin and Milikowski, 1978). In the present study, two dimensional gel electrophoresis in 9% gels (Figures 6, 8 - 10) identified about 150 peptides and well over 300 peptides were counted when 7 to 24 per cent gradient second dimension SDS gels were used (Figure 19).

Spectrin (bands 1 and 2) is an extrinsic protein located on the cytoplasmic surface of the erythrocyte membrane. It migrates on sodium dodecyl sulphate polyacrylamide gels with an apparent molecular weight of 200 000 - 240 000. Its functions include:

i) determining the shape of red blood cells
ii) stabilising of lipid bilayer
iii) contributing to lipid asymmetry
and iv) influencing the distribution of integral membrane proteins.

Abnormalities in spectrin and spectrin-related functions have been reported in Duchenne muscular dystrophy. They include increased phosphorylation of spectrin (Roses et al., 1976), increased osmotic fragility of erythrocytes (Fischer et al., 1976) and reduced deformability (Percy and Miller, 1975). In this study we undertook two-dimensional gel electrophoresis in 12% SDS gel (second dimension gel) to separate high molecular weight peptides of erythrocyte membranes. Forty peptides migrating with molecular weights of between 100 000 and 220 000 were separated. We presumed that most of these comprised of bands 1 and 2 (spectrin). Our results were not able to show any consistent differences between normal control samples and those from Duchenne muscular dystrophy patients and carriers (Figures 11 and 12). The results seem to support the view that spectrin may be normal in Duchenne muscular dystrophy (Falk et al., 1979).

Two different types of transmembrane proteins have been identified:

i) Transport proteins (Band 3)

and ii) Receptor proteins (glycophorins)

Preliminary investigations have reported normal sialoglycoproteins (glycophorins) in muscular dystrophies. The glycophorins are phosphorylated normally in myotonic dystrophy (Wong and Roses, 1979).

Band 3 migrates on SDS polyacrylamide gels with an apparent molecular weight of 90 000 - 100 000. It is associated with spectrin, band 4 and the Na, K, Mg-ATPase activities. Band 3 has been subjected to considerably analysis within the last 10 years. Roses et al. (1975) reported increased phosphorylation of band 3 in Duchenne muscular dystrophy. Subsequently Iyer et al. (1977), Wong and Roses (1979) and Tortolero et al, (1979) reported normal phosphorylation of band 3 in Duchenne dystrophy. In the present study, our two-dimensional protein patterns between 50 000 and 100 000 daltons (Figures 15-17)

could not show any consistent differences between normal control and Duchenne muscular dystrophy samples. Full investigations in our laboratory were plagued by limited sample supplies, we therefore feel that more experiments are needed to confirm this finding. The relation of band 3 to the previously reported abnormal transport of K^+ and Ca^{2+} across membranes has not yet been clearly formulated although the glycoprotein (band 3) is known to affect the transport of ions across membrane-barriers.

Band 5 or red blood cell actin migrates with an apparent molecular weight of 42 000 on sodium dodecyl sulphate gels. Actin polymerisation is induced by the phosphorylated state of spectrin. Abnormalities in the phosphorylation of spectrin may, therefore, affect the function of actin. Up to now there is no evidence that band 5 is abnormal in Duchenne muscular dystrophy. In this report, low molecular weight peptide maps could not show consistent differences between Duchenne dystrophy samples and those from normal controls. Although our protein patterns around 40 000 daltons were not very identical but, differed from sample to sample, the differences seemed not to be related to the diseases under investigation. The observed heterogeneity was most probably due to variations in storage conditions.

FINAL NOTE: Although we did not observe any consistent differences between test and control samples, we would like to point out that full and conclusive investigations were plagued by limited supply of test samples.

7.2 Concluding Remarks

Although it is known that Duchenne muscular dystrophy is the most tragic variety of all the dystrophies and that it is due to an X-linked recessive trait, very little is known about the pathogenesis. At present, identification of carrier-mothers and sex of unborn child and then aborting all male fetuses, seems to be the only way of preventing the disease. This gives little comfort because one-third of the cases is believed to arise from new mutations. Despite immense proliferation of research effort on muscular dystrophies, nobody can boldly say anything useful about HOW and WHERE to search for the fundamental defect. The nature of the basic abnormality in muscle cells which causes them waste is still far from being understood. Nobody understands why some skeletal muscles are affected more than others. The discovery of the basic defect in Duchenne muscular dystrophy may lead to direct cure or at least to effective control of the clinical manifestations. Needless to say that it is equally possible to find an effective cure of some of the symptoms without the knowledge of the nature of the genetic defect.

Although a number of theories including:

i) the myogenic theory

ii) vascular hypothothesis

and iii) neurogenic hypothesis

have been advanced for the pathogenesis of muscular dystrophies, current interest centres on the possibility that there may be a defect in the plasma membrane of muscle and other cells particularly erythrocytes. Unfortunately many conflicting results have emerged from recent investigations. One, nevertheless, wonders whether the observed abnormalities were due to the defect or due to

i) too few samples studied

ii) random controls not properly matched for age and sex

iii) poor experimental designs that could have given alternative interpretations.

Finally, although many investigators have reported abnormalities linked to membrane defects, there are no real grounds for believing that these particular abnormalities are any closer to the gene defect than other defects reported in other systems. The gate to the field of searching for genetic defects remains wide open and the overall feeling for future success is one of optimism.

2 CYSTIC FIBROSIS

INTRODUCTION

1. An Historical Background

Cystic fibrosis (C.F.) is one of the most common genetic diseases in children and young adults in countries with large caucasian populations. The disease manifests itself as a generalised disorder primarily affecting the exocrine glands but the basic defect resulting from the double dose of the defective gene is still unknown.

The saying from the German folklore of the mid-nineteenth century literally translated as:

"The child will soon die whose brow
tasted salty when kissed"

is believed to be the earliest reference to cystic fibrosis (Wood et al., 1976). The genetic nature of the disease was probably first noticed by Garrod and Hurtley (1912) who ascribed Mendelian recessive inheritance to familial congenital steatorrhea which was probably cystic fibrosis. The term 'cystic fibrosis of the pancreas" later shortened to "CYSTIC FIBROSIS" was first used by Andersen (1938). Two years before, the disease had been described by Fanconi (1936) following the recognition of the association between congenital cystic pancreatic fibrosis and bronchioectasis. Other names were proposed for the disorder but the most popular of these, "MUCOVISCIDOSIS" proposed by Farber (1945) because of the observation of increased viscosity of mucous secretions is sometimes still used today.

2. Clinical and pathogenetic manifestations

2.1 Gastro-intestinal system

In more than 80 per cent of the children with cystic fibrosis, the

initial gastrointestinal manifestation is the malabsorption of fat and protein as a result of pancreatic insufficiency (Redmond, 1980). This is a consequence of the progressive destruction of the pancreas which occurs in this disorder. After about 2 years of age, the pancreas of a patient may be unrecognised except as islets clustered in fibrous or adipose tissue. Deficiency of pancreatic lipase, trypsin, chymotrypsin and carboxypeptidase as a consequence of pancreatic destruction and increased bile acid loss (Smalley et al, 1978) result in marked azotorrhea and steatorrhea. The treatment of malabsorption is primarily palliative and is accomplished by pancreatic enzyme replacement associated with low fat and high protein diet with vitamins A and D supplements.

Twenty per cent of the patients with cystic fibrosis are characterised by dilatation of small intestine filled with thickened meconium ileus. The colon is narrowed but returns to normal after relief of obstruction. During the first year of life, most patients produce large foul stools, have ravenous apetites but poor weight gain (Lippe, 1980). Rectal prolapse due to loss of weight, passage of bulky stools and increased abdominal pressure, is apparent in 20 per cent of untreated patients in the first three years of life. Some patients show disaccharide intolerance and deficiency of intestinal lactase (Bakken and Motzfeldt, 1979). Progressive biliary cirrhosis is detectable clinically (Schuster et al., 1977) and histologically at all ages from late intra-uterine life until adult life (di Sant Agnese and Blanc, 1956; Oppenheimer and Esterley, 1975). Increased incidence of diabetes mellitus has also been reported in older patients (Lippe et al, 1980).

2.2 Respiratory system

Over 80 per cent of the cystic fibrosis patients reveal themselves with recurrent infection of the respiratory tract in the first year of life (Dinwiddie, 1980). This pulmonary involvement is characterised by wheezing, coughing and stertorous breathing. Lungs become blocked with infected mucus. Excess mucus results from an imbalance between rate of production and rate of clearance (Wood et al., 1975), the former outstripping the latter. Respiratory insufficiency and atelectasis (lung collapse) may be the consequences of the excess mucus. In about 90 per cent of the patients, Staphylococcus aureus and Pseudomonas aeruginosa invade the respiratory tract (Stern et al., 1978) and the organisms are believed to cause atelectasis (Lloyd-Still et al., 1974) by producing phospholipases which act on pulmonary surfactant. Progression of pulmonary involvement becomes characterised by decreased exercise tolerance, chronic productive cough, tachypnea and clubbing of the fingers.

Treatments of the affected respiratory system are by physiotherapy and administration of antibiotics. Anti-Staphylococcal agents such as flucloxacillin are normally used to try to clear the respiratory tract of infections. Death of cystic fibrosis patients frequently is the result of pneumonia, anoxia and exhaustion after long periods of respiratory insufficiency.

2.3 Exocrine glands secretions - Eccrine sweat and saliva

The increased sodium and chloride concentrations of eccrine sweat, first described by di Sant'Agnese et al. (1953), is the most constant abnormality in cystic fibrosis. This observation has become the

cornerstone of all the diagnostic methods of the disease. The most reliable method of the "sweat test" used in diagnosis is the pilocarpine iontophoresis method of Gibson and Cooke (1959).

TABLE IV ELECTROLYTES IN EXOCRINE SECRETIONS

SECRETION	ELECTROLYTES			REFERENCES
	Na^+	Cl^-	K^+	
Sweat (mol/l)				
CF	154.5	149.9	15.1	Hamdi I et al Monogr Pediatr 10 (1979) 84
Control	43.6	33.2	10.6	
Submaxillary Saliva (mEq/l)				
CF	46	25	23	Mandel I et al Amer.J.Dis. Child 113 (1967) 431
Control	30	17	20	
Tears (mM/l)				
CF	137	133	23	Botello S et al J. Pediat 83 (1973) 601
Control	140	26	26	

The above Table clearly shows the abnormally high concentrations of sodium and chloride in sweat and submaxillary saliva of cystic fibrosis patients. The electrolytes have also been found to be elevated in parotid saliva from cystic fibrosis patients (Hain J. et al., 1979). It was postulated that the abnormality could result from:

i) abnormal concentrations of ions in the primary secretions of a gland,

ii) abnormal handling of ions by the duct,

and iii) abnormal permeability of the duct to water.

Experiments by Kaiser et al. (1970) and subsequently by Mangos (1973) strongly suggested the presence of a factor in sweat of cystic fibrosis patients that decreased the net transductal reabsorption and back-diffusion of sodium and chloride resulting in abnormally high concentrations of these ions in secreted sweat. The relation of this factor to humoral factors demonstrated by ciliastatic bioassays is not clear.

In saliva a sodium transport inhibitory factor was demonstrated by Mangos (1973) and Taylor et al (1974). The factor was found:

i) not to be linked to $Na^+ + K^+$ ATPase

ii) not to be eliminated by dialysis

and iii) to be mimicked by positively-charged polycations such as polylysine and polyornithine.

From these characteristics it was postulated that the factor was a positively charged macromolecule. The relation of this factor to the positively charged macromolecule subsequently demonstrated by isoelectric focusing (Wilson and Fudenberg, 1975) of cystic fibrosis serum is not very clear.

3. INCIDENCE AND GENETICS

There are four criteria for the diagnosis of cystic fibrosis:

(1) A positive sweat test (sweat chloride and sodium greater than 60mmol per litre - Gibson and Cooke, 1959).
It is perhaps worth to note that there are other conditions such as adrenal insufficiency, fucosidosis and nephrogenic diabetes insipidies that are also associated with raised levels of sodium and chloride.

(II) Chronic obstructive pulmonary disease. This is present in almost all cases and it is characterised by persistent dry cough between attacks of respiratory infection and throat clearing.

(III) Exocrine pancreatic insufficiency occurs in 80-90 per cent of the patients. Evidence of pancreatic insufficiency or dysfunction is provided by:

(a) Increased content of albumin in meconium to levels greater than 20mg per gram of meconium.

(b) Reduced chymotrypsin, usually below 120 g per gram of stook.

(c) Severe steatorrhea (10-50g fat per day compared to 4g fat per day in normal controls).

(IV) Family History.

In addition, normal bicarbonate levels in stools is generally regarded as indicative of the absence of cystic fibrosis (Hardon et al, 1968).

In countries with predominant caucasian populations, a patient with cystic fibrosis is born every 1500-2000 life births (Nevin, 1978; Prosser, 1979). The racial predilection of the disease is clearly

evident in Table V below.

The disease is inherited as an autosomal recessive trait (di Sant' Agnese and Davis, 1976; Super, 1979) expressed only in the homozygous state without X-linkage, hence boys and girls are affected equally although males may predominate later as a result of higher mortality in females (Stern et al, 1976; Gurwitz et al, 1979). A family with a case of the disease has 1 in 4 chance of each future child being affected or normal and 1 in 2 chance of being a carrier.

Studies have provided evidence of normal karyotype in cystic fibrosis (Blanc et al, 1968). The trait is not linked with the ABH blood groups (Di Matteo et al, 1979) or the major histocompatibility complex.

TABLE V

INCIDENCES OF C.F.

LOCATION	INCIDENCE	REFERENCES
Washington DC (Blacks)	1:17000	Kulczycki & Schauf Amer.J.Dis.Child 127 (1974) 64.
England (General population)	1:2000	Dinwiddie J-J Mart Child Health 4 (1979) 370.
England (Pakistani descent)	1:10 000	Goodchild et al Arch.Dis.Child 49 (1974) 739.
Namibia (Dutch descent)	1:635	Super-Monogr Pediatr. 10 (1979) 106.

4. STUDIES IN SEARCH OF BASIC BIOCHEMICAL DEFECTS IN CYSTIC FIBROSIS

4.1 Tissue Culture

Several studies have been conducted on tissue cultures of fibroblasts from cystic fibrosis patients and from the conflicting results, it is generally consented that fibroblasts are somehow affected by the basic defect in this disorder. Since the reporting of metachromasia in cystic fibrosis fibroblasts (Danes and Bearn, 1969), many other conflicting and disparate results on RNA methylation, chemical composition of membranes and activities of lysosomal enzymes of these cells have been reported.

Evidence on the extent of involvement of fibroblast membranes in cystic fibrosis is conflicting in most instances. Of particular interest is the increased resistance of fibroblasts from cystic fibrosis patients to cytotoxicity of oubain (Epstein and Breslow, 1977), dexamethasone (Epstein et al, 1977) and sex steroids (Breslow et al, 1978). The binding capacity of the drugs to the cells were normal (Breslow et al, 1978). Although these observations suggested abnormal fibroblast plasma membranes, they could not be confirmed in other laboratories (Kurz et al, 1979). Also worthy considering is the greater fluidity of plasma membranes observed in fibroblasts from cystic fibrosis patients (Christian et al, 1977) and increased premature senescence of fibroblasts from cystic fibrosis patients (Shapiro et al, 1979). Although the bulk lipid fluidity as reflected by measurements of temperature-dependent transitions and fluorescence polarisation of 1-acyl-2-(N-4-nitrobenzo-2-oxal,3,diazole) amino caproyl phosphatidyl choline (Christian et al, 1977) was increased, critics have been prompt to point out that the study was conducted on fibroblasts from a single patient

and that the probe used was too big to align itself properly with the membrane lipids. Interestingly, fluorescence polarisation measurements of smaller probes, β-parinaric acid and diphenyl hexatriene (Dale et al, 1977) were unable to show any differences in fluidity between fibroblasts from cystic fibrosis patients and those from control subjects.

The abnormally high cAMP levels observed in isoproterenol-stimulated fibroblasts from cystic fibrosis patients (Buckwald, 1976) seemed to suggest abnormal plasma membranes of these cells. Subsequent investigations suggested abnormal β_2-receptor because propranolol, a β_2-blocker, inhibited the isoproterenol-stimulated accumulation of cAMP (Roscher et al, 1979).

The biochemical analysis of plasma membranes has been trailing behind, presumably because of lack of adequate methods of isolating pure fibroblast plasma membranes. The traditional and most widely used methods based on the original procedure by Warren et al (1966) which employed fixation of cell surface with heavy metal e.g. Zn prior to disruption, produced low yields of plasma membranes heavily contaminated with intracellular organelle membranes. With the recent introduction of a new method of isolating fibroblast plasma membranes (Kartner et al, 1977), the plasma membranes have been analysed for both lipid and protein compositions. No significant differences in phospholipid and fatty acid (Riordan and Buckwald, 1979) and protein compositions have been found between cystic fibrosis fibroblasts and those from controls.

It is perhaps worth mentioning the findings of Hosli et al. (1977) that alkaline phosphatase was elevated in cystic fibrosis fibroblasts and that this could be induced by Tamm-Horsfall glycoprotein. Based

on these observations they postulated that Tamm Horsfall glycoprotein had an abnormal effect on lyposomes of cystic fibrosis fibroblasts resulting in release of alkaline phosphatase, a process that affects intracellular digestion.

4.2 Humoral factors

The search for gene products in cystic fibrosis led Spock et al. (1967) to make an exciting observation that when serum from cystic fibrosis homozygotes was added to cultured explants of rabbit trachea, the synchronous beating of the trachea cilia rapidly became disorganised. Control serum had no effect. Subsequently, similar inhibitions of ciliary motion were demonstrated in oyster gills (Bowman et al., 1969) and in other ciliated systems such as fresh water mussel gill, human sperm and ciliated protozoa. The ciliastatic bioassay-systems were further extended by Cohen and Daniel (1974) who demonstrated that the motile bacterium, Proteus vulgaris was rapidly agglutinated by small samples of cystic fibrosis serum. The factors presumed responsible for ciliary inhibition have become known as cystic fibrosis dyskinesis factors or cystic fibrosis factors (CFF). The factors have been demonstrated in many body fluids and cell cultures from cystic fibrosis homozygotes and heterozygotes. The basis for ciliary cessation after exposure to cystic fibrosis serum is yet unknown but experiments have suggested that this might be connected with the increased mucus production (Conover and Conod, 1978) and that the process leading to cessation of ciliary beat might be linked with the observed elevated uptake of calcium by ciliated epithelium (Bogart et al, 1979).

The molecular identities of cystic fibrosis factors are as yet unclear but they appear to be closely associated with IgG_1 (Bowman

et al, 1975). Because of lability of these factors, only some of their properties have been determined. The factors have been found to have molecular weights of 1 000 - 10 000, with net positive charge. The factors are heat labile and present in both homozygotes and heterozygotes despite the fact that heterozygotes show no symptoms. This makes these factors unlikely to be directly linked with the secondary effects of the disease (Nagy et al, 1979).

Using isoelectric focusing, Wilson and Fudenberg (1975) were able to demonstrate a cationic protein of pI 8.4 in serum samples from cystic fibrosis homozygotes and heterozygotes. The relation of this cystic fibrosis protein to the mucociliary inhibitors reported by other workers is not yet clear. This cationic protein may be somehow linked to abnormally elevated polyamines observed in blood of cystic fibrosis patients (Baylin et al, 1980). Recently Wilson and Bahn (1980) have reported that peripheral leucocytes produced three ciliary dyskinesia substances that could be separated by Biogel P-10 chromatography. They suggested that one of the fractions (Fraction III), molecular weight 9 000, could be C3a because it could be removed from supernatants by antisera raised against C3a. This observation agreed with earlier reports by Conover and Conod (1973) that the mucociliary inhibitor could be C3a. It is perhaps worthy mentioning at this stage, that some ciliary dyskinesis factors have also been reported in sera of patients with a variety of other diseases (Conod and Conover, 1973). It is not yet clear whether the factors are normal products that accumulate in disease state because of lack of normal degradation or they are abnormal proteins.

Alpha-2-macroglobulin (α_2M) is one of the major components of a heterogenous group of serum proteins collectively known as the alpha globulins. It is a glycoprotein, molecular weight 725 000, that

regulates a variety of proteolytic enzymes which participate in inflammatory and homeostatic events. The molecule can be dissociated into four equal subunits of molecular weight 185 000 when incubated with a reducing agent, dithioerithreol at 37°C. When the subunits are heated at 90°C for 30 minutes in SDS and mercaptoethanol, two peptides of molecular weights 125 000 and 62 000 are produced.

Alpha-2-macroglobulin is capable of binding proteinases such as trypsin, thrombin and cathepsin B1, lectins like concanavalin A and phytohemagglutinin and some metal ions e.g. zinc, nickel. It is also believed to inhibit chemotactic factors e.g. C3a. α_2M is unique among the naturally occurring protease inhibitors as it binds to the site other than the active site, thereby preserving the catalytic potential of the protease while at the same time efficiently masking enzyme activity against protein substances. The protein-protein complex formed between α_2M and its enzyme is resistant to dissociation.

α_2M and alpha-1-antitrypsin (α_1AT) are the major protease inhibitors in human serum. α_2M differs from α_1AT in its mechanism of inhibiting trypsin or other proteases. The trypsin-α_2M complex retains enzymatic activity against substances of low molecular weights whereas the trypsin-α_1-AT complex is enzymatically inactive. Abnormalities in the interactions between α_2M and proteases have been reported in cystic fibrosis. Wilson et al (1976) reported a deficiency of an α_2M-protease complex in plasma of patients with cystic fibrosis and in obligate heterozygotes. They suggested that an abnormality in the binding capacity of α_2M to plasma proteases might account for the presence of cystic fibrosis factors. The argument behind such a suggestion was that α_2M-protease complex retains its activity towards low molecular weight substrates, therefore its absence results in the appearance of low molecular weight peptides. Subsequent

and supportive experiments by Shapira et al (1976) demonstrated a decreased binding of endopeptidases to α_2M from patients with cystic fibrosis.

Experiments by Ben Yoseph et al (1979) compared the carbohydrate composition of purified α_2M from control, cystic fibrosis homozygotes and heterozygotes. They obtained normal amounts of total hexose but as much as 40% decrease in sialic acid in α_2M from cystic fibrosis patients. They then compared the binding of constant amounts of α_2M preparations to increasing amounts of agarose-bound concanavalin A. The binding was substantially decreased in α_2M preparations from cystic fibrosis patients. From this they postulated that the mutant form of α_2M was probably a result of mutation in one of the enzymes that synthesised α_2M and that this led to a different extent of branching of the oligosaccharide moiety of this glycoprotein.

Recent experiments by Romeo et al (1980) have not been able to show any differences between cystic fibrosis patients and controls in the binding of α_2M to trypsin and also some protease subunit cleavage experiments were unable to show any differences between cystic fibrosis α_2M preparations and those from normal controls (Burdon, 1980). Comings et al (1980) using two dimensional electrophoresis were unable to show any mutant forms of α_2M in cystic fibrosis thus extended the view that α_2M might be normal in this disorder.

In this report we attempted to show some cystic fibrosis proteins in sera from cystic fibrosis patients by the isoelectric focusing method of Wilson et al (1977). We also searched for some cystic fibrosis gene products in whole serum by two dimensional electrophoresis as described by Anderson and Anderson (1977). An attempt has been made here to compare peptide maps of purified α_2M from cystic fibrosis patients and control subjects as analysed by two dimensional gel electrophoresis.

5. MULTIPLE SCLEROSIS (MS)

5.1 General Overview

Multiple sclerosis is an inflammatory demyelinating disease found in a variety of clinical forms ranging from an exacerbating-remitting course to a chronic progressive disease. Epidemiologic studies have uncovered important clues about multiple sclerosis but the cause remains unknown. The picture on aetiology is further complicated by extremely varied clinical patterns. Risk areas have been divided into high (50 - 100 cases per 100 000 people) and low (5 - 10 cases per 100 000 people) risk areas. A relation exists between prevalence of multiple sclerosis and latitude of birthplace with very low incidences in the tropical areas. The risk of developing symptoms is age dependent with a peak at the age of 30 years and few cases occuring before the age of 15 years or after the age of 60 years. The disease is very rare among black Africans and Orientals.

A consistent finding in several studies of multiple sclerosis patients has been the slightly but persistently elevated serum and cerebrospinal fluid antibody titres to measles and less commonly to other viruses such as vaccinia, mumps and herpes simplex (Haire and Hadden, 1970). These findings suggested that multiple sclerosis was a virus disease with exacerbations and remissions reflecting emerging antigenic variants.

Another established finding is the induction of inflammatory demyelinating disease, relapsing experimental allergic encephalomyelitis, in immunodeficient animals immunised with central nervous system tissue or myelin basic protein. The finding suggests that MS is an autoimmune disease directed against central nervous system antigens. The difficulty in trying to link relapsing experimental allergic encephalomyelitis (REAE)

and multiple sclerosis is that there is no convincing evidence of autoimmunity to CNS antigens in patients with multiple sclerosis.

The histopathologic picture in multiple sclerosis is that of demyelinated lesions closely resembling those of cell mediated immune reactions. The early active lesions consist of inflammatory demyelination with mononuclear exudates. Older lesions consist of plasma cells, mature lymphocytes and astrocytes. Notably, lesions follow the contours of the third and fourth ventricles and sulci of cerebral hemispheres. The lesions that fuse together to form plaques contain increased proportions of immunoglobulins that are produced locally. These immunoglobulins appear in the CSF and they are characterised by increased kappa to lambda ratio (Olhsson and Link, 1973). When subjected to electrophoresis, these immunoglobulins show characteristic discrete or "oligoclonal" pattern of bands (Johnson and Nelson, 1977). This finding is used as one of the tests for the diagnosis of multiple sclerosis. It is perhaps worthy mentioning that other diseases e.g. neurosyphilis also show similar banding pattern, but this disappears with time.

Familial cases are not uncommon and the risk of a first degree living relative of having the disease is 10 - 15 times higher than in the rest of the population. Significant genetic associations of MS with HLA-A3, HLA B7 and HLA-DW2 have been described.

Recent observations have shown reduced capabilities of lymphocytes from MS patients to produce interferons and to have reduced natural killer cell activity (Benczur et al, 1980). These observations seem not to be specific for MS because they have been demonstrated in other viral diseases.

5.2 Humoral Factors in Multiple Sclerosis

Antibodies to specific myelin basic protein (MBP), measles and other viruses are found elevated in both serum and cerebrospinal fluid of multiple sclerosis patients. A source of considerable confusion, though, is that MBP and measles virus antibodies cross-react in some serological tests. One interesting observation was that when MS patients' sera were added to myelinated cultures of nervous tissue, myelin was destroyed and myelination inhibited. The responsible "factors" have not been fully characterised but they are presumed to be immunoglobulins or enzymes.

A factor that inhibits ribonucleic acid (RNA) synthesis in lymphocytes from normal donors has been demonstrated in sera of MS patients. The factor is also known to depress RNA synthesis in lymphocytes from multiple sclerosis patients, although to a lesser degree than in lymphocytes from normal donors. The factor appears most frequently during acute attacks of the disease (Stjernholm et al., 1970) and blast cells seem more sensitive to the factor than resting cells (Burns et al., 1971). Purification of the factor on Sephadex G100 indicated that the factor is comparable in size to human albumin, non-dialyzable, stable to prolonged heating in serum but could be inactivated by ammonium sulphate precipitation or DEAE-cellulose chromatography (White et al., 1975). Recent reports (Anderson et al., 1979) have shown that MS serum factor inhibits RNA, DNA and protein synthesis in lymphocytes stimulated by PHA, ConA or PWM to undergo blast transformation.

Crossed immunoelectrophoresis and immunoelectrofocusing experiments have recently revealed that α_2M from MS patients sera was abnormal (Rastogi and Clausen, 1980). The relation between this finding and the serum "factors" that inhibit lectin-induced RNA, DNA

and protein synthesis is not yet clear although α_2M is known to:

(i) bind proteases that play positive role in transformation processes of lymphoreticular cells

and

(ii) interact with various lectins. The interaction inhibits mitogen response to lectins of lymphoreticular cells.

In this report MS samples were analysed by two dimensional electrophoresis in an attempt to reveal MS serum "factors". α_2M preparations from these samples were also characterised by (i) Immunoelectrofocusing and (ii) Electrophoresis.

EXPERIMENTAL

6.1 Materials

(a) 5.13 M urea

30.8g urea

q.s. 100ml with distilled water

(b) Stock acrylamide for isoelectric focusing

33g acrylamide

1g methylene bisacrylamide

q.s. 100ml with distilled water

(c) Fixing solution

75g trichloroacetic acid (TCA)

25g sulphosalicyclic acid (SSA)

150ml ethanol

25ml glacial acetic acid

325ml water

(d) Destaining solution

300ml ethanol

650ml water

50ml glacial acetic acid

(e) Anode solution (Anolyte)

5ml TEMED

q.s. 100ml with double distilled water

(f) Cathode solution (catholyte)

1ml 85% (w/v phosphoric acid

q.s. 30ml with double distilled water

(g) Staining solution

0.5g Coomassie blue R250

225ml ethanol

225ml water

50ml glacial acetic acid

(h) 0.25M sodium phosphate buffer pH7.5

3.55% (w/v) $Na_2HPO_4 \cdot 2H_2O$

adjusted to pH7.5 with

3.9% (w/v) sodium dihydrogen phosphate

(i) 5mg/ml chloramide-T solution

0.5g chloramide-T

q.s. 100ml with 0.05M sodium

phosphate buffer pH7.5

(j) Fixing solution for silver staining

500ml methanol

120ml glacial acetic acid

380ml water

(k) Gel expanding solution

100ml ethanol

50ml glacial acetic acid

850ml water

(l) Paraformaldehyde solution

800ml water

40g paraformaldehyde

14.3g sodium cacodylate adjust to pH7.3
q.s. 1000ml with water

(m) Cupric nitrate-silver nitrate solution

100ml distilled water

3.5g silver nitrate

1.5ml of 0.5% (w/v) cupric nitrate

4.0ml pyridine

8.0ml absolute ethanol

(n) Diamine solution (made within 5 minutes of use):

30ml of 19.4% (w/v) silver nitrate

22.5ml sodium-ammonium hydroxide mixture*

55ml of 20% (v/v) ethanol

*sodium-ammonium hydroxide mixture

100ml of 36% (w/v) sodium hydroxide

45ml concentrated ammonium hydroxide

(o) Reducer I

100ml absolute ethanol

6ml of 1% (w/v) citric acid

2.5ml of 3.7% (v/v) formaldehyde

q.s. 1000ml with water

(p) Reducer II

100ml absolute ethanol

5ml of 1% (w/v) citric acid

5ml of 3.7% (v/v) formaldehyde

q.s. 1000ml with distilled water

(q) Photographic reducer

50ml solution A

50ml solution B

200ml distilled water

Solution A

37g sodium chloride

37g cupric sulphate

850ml water

Few drops of conc. ammonium hydroxide until the blue ppt redissolved.

q.s. 1000ml with distilled water

Solution B

436g sodium thiosulphate

q.s. 1000ml with water

(r) 0.12M barbitone buffer pH8.6

24.7g sodium barbitone

0.2g sodium azide

800ml double distilled water

Adjust to pH8.6 with 1M HCl

q.s. 1000ml with double distilled water

(s) 2% agarose

2g agarose

0.01 sodium azide

q.s. 100ml with double distilled water.

6.2 Methods

6.2.1 Processing of serum samples

Venous blood, obtained from control, cystic fibrosis patients and multiple sclerosis sufferers was allowed to clot for 4-6 hours at 4°C. The samples were then centrifuged at 5000g for 10 minutes at 4°C. The serum samples were divided into 0.5ml aliquots and kept frozen at -70°C.

6.2.2 Isoelectric focusing

Isoelectric focusing in thin layer polyacrylamide gels using the LKB multiphor was performed according to the method of Wilson et al (1977). The gel mixture employed was made up of:

9ml stock acrylamide solution (b)

0.3ml of 5% (v/v) TEMED solution (e)

50ml of 5.13M urea

2.9ml ampholines pH3.5-10

0.1ml ampholines pH2.5-4

The mixture was filtered and then degassed and then 0.2ml of fresh 10% (w/v) ammonium persulphate was added to achieve polymerisation. The gel mixture was poured into the gel mould made up of three glass plates as described in LKB application note 250. The gel was allowed to set for 1 hour at room temperature and for 30 minutes at 4°C. After setting, the edges of the gel were released from the thick glass plate. The plates were then separated and the thin glass plate supporting the gel was sealed on the cooling plate of the multiphor by 1% (w/v) Triton X-100.

Prefocusing was performed for 30 minutes at 350 volts, constant voltage, using 5% (v/v) TEMED in double distlled water as the catholyte

and 1/30 dilution of 85% (w/v) phosphoric acid as anolyte. Samples were then applied on 3MM whatman chromatography pads (7mm x 10mm) placed about 5mm from the anodic strip. Electrofocusing was performed at 35 watts, constant power, for 1 3/4 hours. The sample pads were not removed until the end of the run. The temperature of the cooling water was kept between 4-6°C.

6.2.3 pH gradient measurement

The pH gradient was measured by surface pH electrode, after the run. To avoid errors caused by edge effects, the pH measurements were done in the centre of the gel where no samples were applied.

6.2.4 Fixing, staining and destaining of focused gels.

After focusing, the gels were fixed by placing them overnight in fixing solution (c). The gels were then agitated for 2 hours in staining solution (g). Fixing and staining were sometimes done simultaneously for 30 minutes over a boiling water bath in a solution consisting of:

135g sulphosalicyclic acid
450g trichloroacetic acid
4.5g Coomassie blue R250
2790ml water
1350ml methanol

Destaining of gels was done in several changes of destaining solution (d).

6.2.5 Selective radioiodination of proteins in thin layer polyacrylamide gels

In an attempt to increase the sensitivity of visualising proteins without interfering with their isoelectric points, a method of

labelling proteins after focusing in thin layer polyacrylamide gels was devised. The method employed was a modification of the chloramine-T method first described by Greenwood and Hunter (1963).

A 10µl aliquot of $Na^{125}I$ solution, approximate activity 1.0mCi, in 0.25M, phosphate buffer pH7.5, was dispersed into a disposable tube. 10µl of 5mg/ml chloramine-T were added. The mixture was applied on selected points of gel strip cut off from the focused thin layer polyacrylamide gel slab along the pH gradient. After 10 minutes the gel strip was transferred into an SDS sample buffer consisting of:

2g SDS

2ml 2-mercaptoethanol

10g glycerol

q.s. 100ml with distilled water

After 30 minutes the gel strip containing labelled proteins was sealed onto SDS slab gel for second dimensional run. 1.2mg/ml sodium metabisulphite was sometimes used instead of SDS sample buffer to reduce excess chloramine-T and free iodine. The second dimension SDS gel electrophoresis was performed as described earlier in Part 1. After this run the gel was dried and auto-radiographied for 1 week.

6.2.6 Two dimensional gel electrophoresis of serum samples

This was done as described in Part 1 with the following exceptions:

(1) The isoelectric focusing gel contained 0.05ml pH2.5-4 ampholines and 0.45ml pH3.5-10 ampholines

(2) Linear gradient SDS gels (7-24%) were used.

The Coomassie blue staining was performed as described earlier. The silver staining was also performed.

Silver Staining:

The procedure was carried out essentially according to the method described by Switzer et al (1979) as follows:

(i) The gel was left overnight in fixing solution (j). The gel shrank in this solution.

(ii) To expand the gel to its original size, it was placed in expanding solution (K) for 2 hours.

(iii) The gel was then washed for 15 minutes in three changes of 10% (v/v) redistilled ethanol.

(iv) The gel was then agitated for 30 minutes in paraformaldehyde solution (l).

Paraformaldehyde solution was prepared by adding 40g paraformaldehyde to 800ml water at 60°C. A few drops of sodium hydroxide were added with stirring to facilitate dissolution. 14.3g of sodium cacodylate were added and then the solution was adjusted to pH7.3 with 1M HCl. The total volume was then made to 1000ml with double distilled water.

(v) The gel was then washed for 15 minutes in three changes of 10% (v/v) redistilled ethanol.

(vi) The gel was left in cupric nitrate/silver nitrate solution (m) for 30 minutes.

(vii) The gel was then left for 10 minutes in diamine solution (n) prepared just before use and disposed of immediately after use.

(viii) The gel was agitated for 2 minutes in reducer I and for 30 minutes in 5 changes of reducer II.

(ix) Background staining was removed by placing the gel for few seconds in photographic reducer (q). The gel was finally washed with water and photographed for permanent record.

6.2.7 Preparation of alpha-2-macroglobulin

The preparation of alpha-2-macroglobulin was done essentially according to the method of Barrett et al (1979). The procedure used was as follows:

(i) 20ml of blood were collected into glass centrifuge tubes and allowed to clot at 4°C for 4-6 hours.

(ii) The serum was obtained by centrifuging blood at 5000g for 10 minutes.

(iii) The serum was collected and stored at -20°C until required.

(iv) 12ml of serum were mixed with 3.4ml of 25% (w/v) polyethylene glycol 6000, pH 6.5 and left to stand for at least 30 minutes at 20°C.

(v) The precipitate formed was centrifuged at 10 000g for 10 minutes and discarded.

(vi) The supernatant was mixed with 8.6ml of 25% (w/v) polyethylene glycol 6000 and left for 30 minutes at room temperature.

(vii) The precipitate formed was centrifuged at 10 000g for 10 minutes and the supernatant was discarded.

(viii) The precipitate was dissolved in 0.05M Tris-HCl buffer pH8.0 to a total volume of 6.0ml. This fraction was run on Blue Sepharose 6B column (3.5 x 40cm) in the same buffer and 2.5ml fractions were collected at a rate of 10ml per hour. The protein peaks were detected by measuring absorbances of the fractions at 280nm. Fractions containing the least contaminated α_2M were detected by gel electrophoresis. These fractions were pooled and concentrated. Concentration was achieved by sealing the pooled fractions in a 1cm diameter dialysis tubing and then covering the tubing with polyethylene glycol 6000. This was done at 4°C for 1 hour. The resulting concentrated solution was dialysed at 4°C for 24hrs against 1% (w/v) glycine pH6.5 to precipitate immunoglobulins

The dialysed solution was spun at 20 000g and the precipitate formed was discarded. The supernatant was concentrated as described above to a volume of $\frac{1}{2}$ the starting volume. The resulting α_2M solution was analysed by isoelectric focusing, SDS electrophoresis, two-dimensional gel electrophoresis and immunoelectrofocusing.

6.2.8 Immunoelectrofocusing of α_2M preparations from CF, MS and control samples

Immunoelectrofocusing combines electrofocusing and immunoelectrophoresis. Electrofocusing was done as described above for serum samples.

Immunoelectrophoresis:

Plates containing anti-α_2M antibodies were prepared as follows:

(i) 2g agarose and 0.01g sodium azide were made up to 100ml with double distilled water. The mixture was heated over a water-bath until all the agarose had completely dissolved.

(ii) 3.5ml of the above 2% agarose was mixed with an equal volume of 0.12M barbitone buffer pH8.6 at 60°C. The mixture was allowed to cool to 60°C and then 100μl of anti-α_2M antibodies were added.

(iii) The mixture was poured on to a clean glass plate (8cm x 8cm) and allowed to set.

(iv) A strip of gel 1cm wide containing the focused α_2M was laid on the agarose along one edge of the glass slide.

Immunoelectrophoresis was performed for 18hrs from cathode to anode using 0.06M barbitone buffer pH8.6 as the electrode solution. After the run, the agarose gel was squeezed by applying pressure on filter papers covering the gel. The filter papers were peeled off and the gel dried under a current of warm air. The gel was then stained for 1 hour in Coomassie blue solution made up of:

5g Coomassie blue R

450ml 96% ethanol

100ml acetic acid

450ml double distilled water

Destaining was done in 2-5 minutes in solution consisting of

250ml of 96% ethanol

100ml acetic acid

450ml double distilled water.

RESULTS

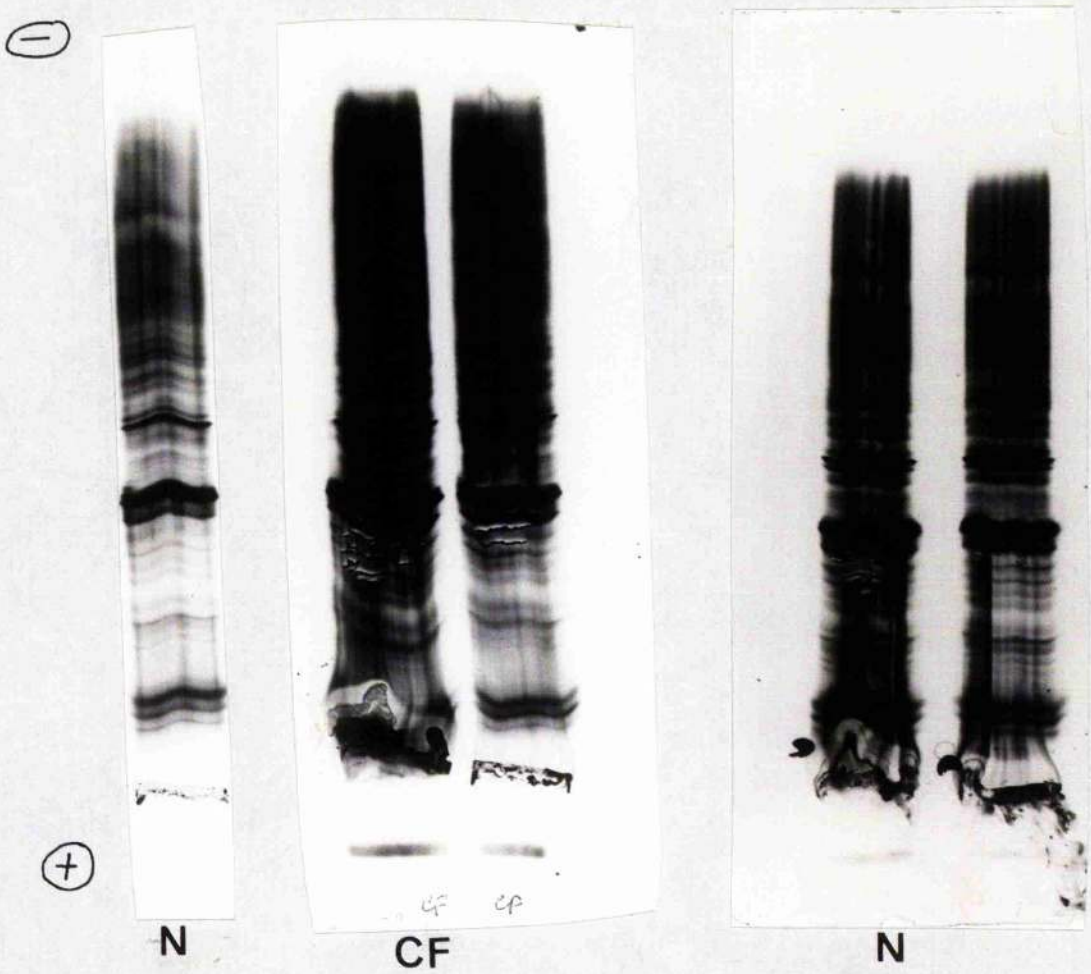


Figure 21 Results of isoelectric focusing of whole serum in a pH 2.5 - 10 gradient. N, normal control; CF, cystic fibrosis homozygotes. The anode + and the cathode - ends are shown.

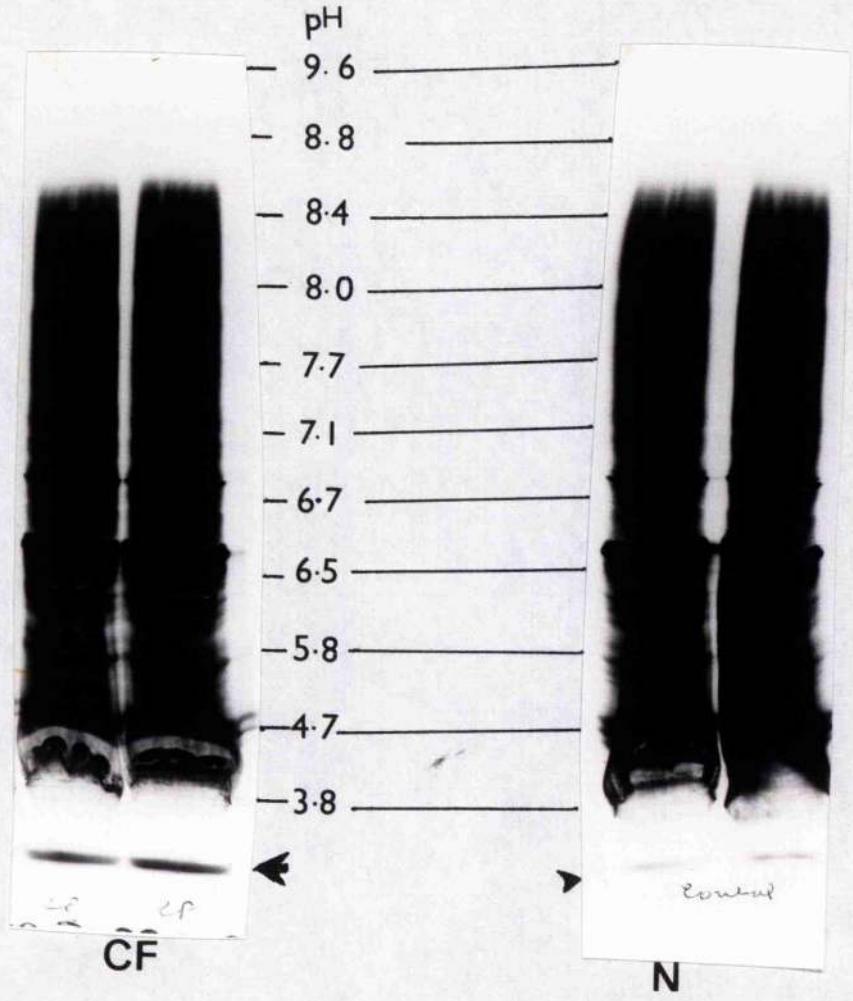


Figure 22 Results of isoelectric focusing of whole serum from cystic fibrosis patients and normal control subjects. 20μl samples were run. The pH gradient values as measured by surface electrode are shown.

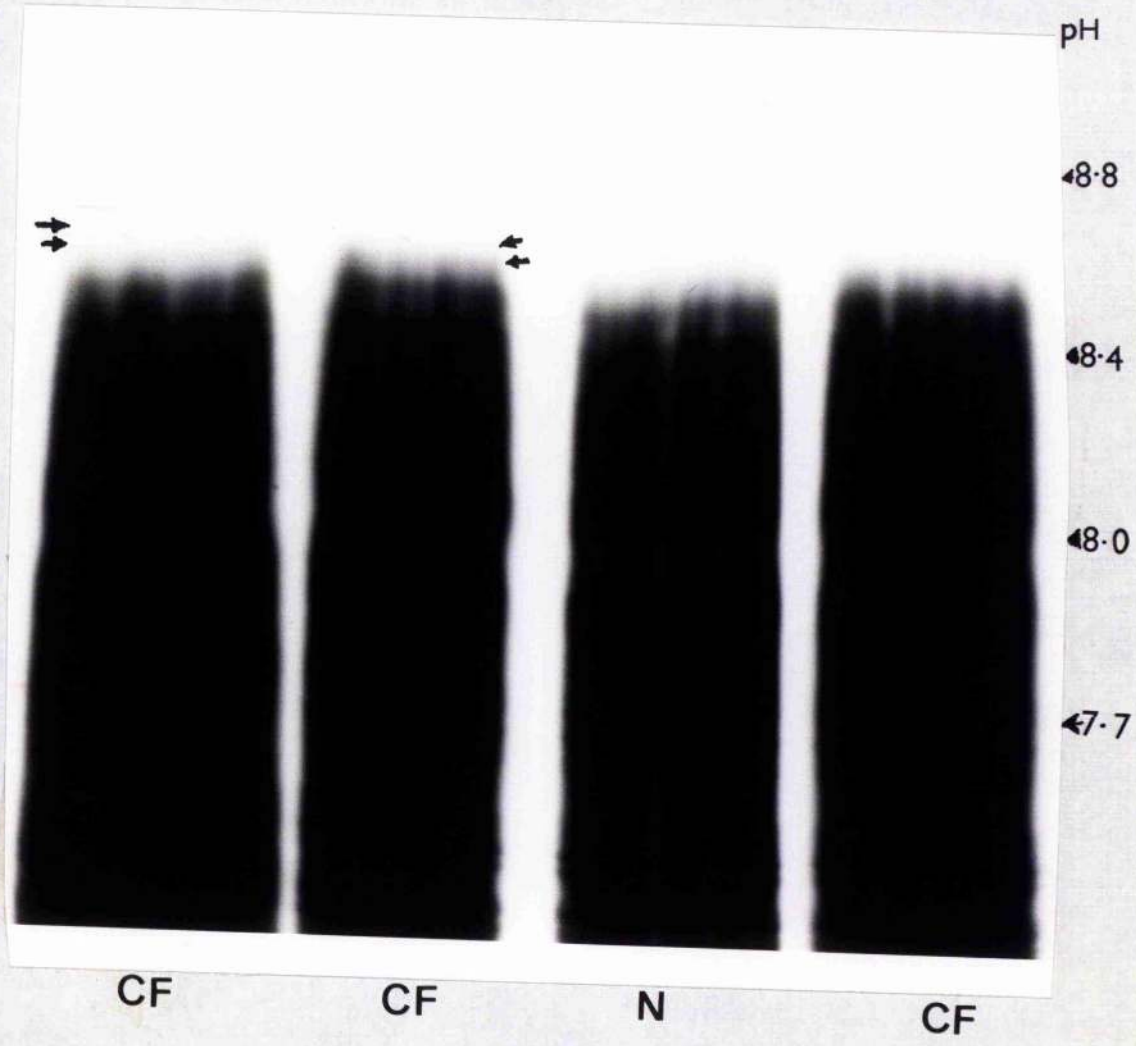


Figure 23 Enlargement of part of the cathodal region of the polyacrylamide gel with arrows indicating the positions of the cystic fibrosis proteins (CFP).

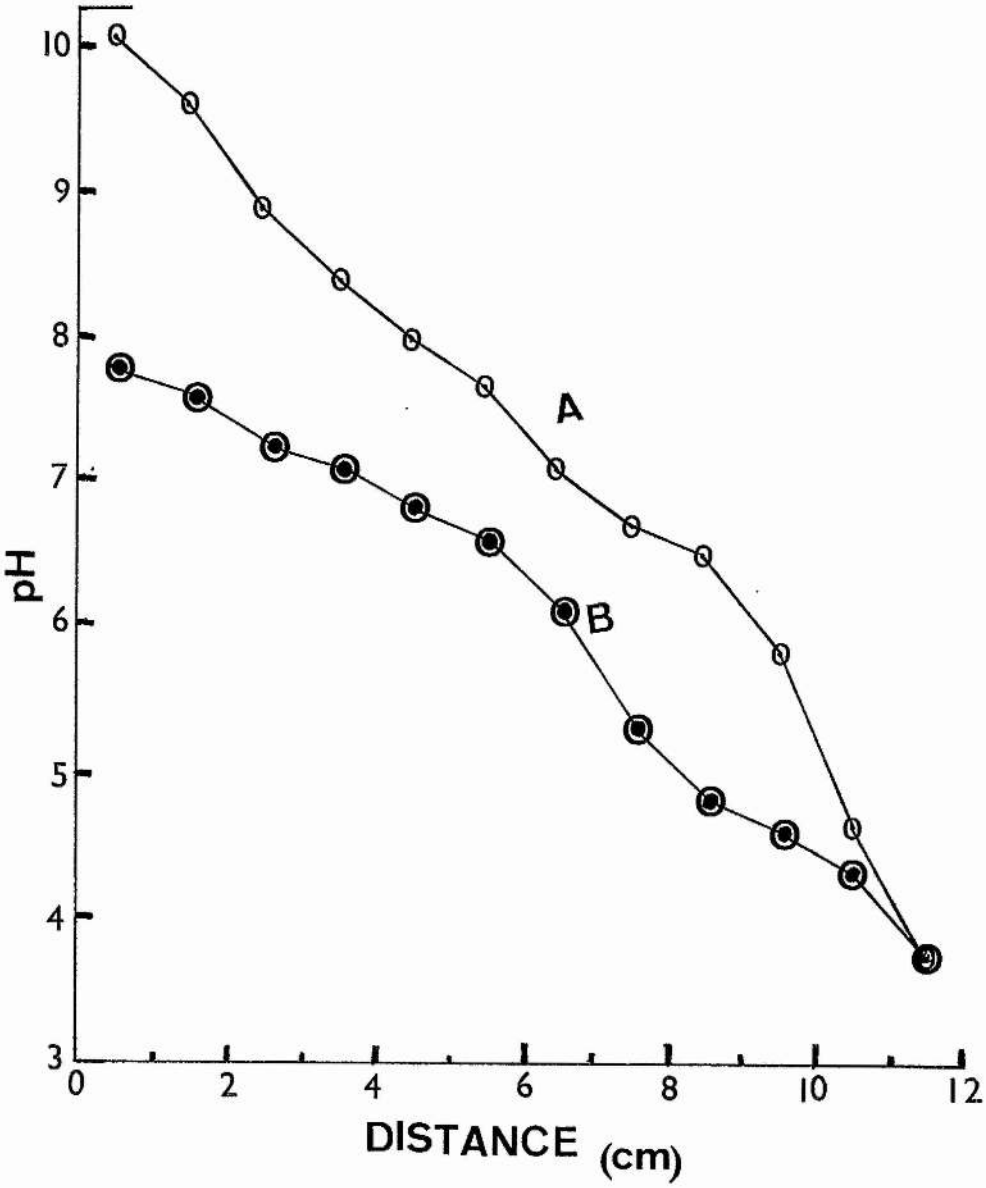


Figure 24 Results of pH measurements from cathode to anode. Curve A is from 0.05ml pH 2.5-4 ampholines 0.07ml pH 3.5 - 10 ampholines in 15ml of gel mixture. Curve (B) is from 0.05ml pH 2.5 - 4 ampholines and 0.45 pH 3.5 - 10 ampholines in 10ml of gel mixture.

TABLE VI

PRESENCE OF CYSTIC FIBROSIS FACTOR IN HOMOZYGOTES AND NORMAL CONTROL SERUM SAMPLES

SERUM TYPE	C.F. HOMOZYGOTE	NORMAL CONTROL	REF
NUMBER TESTED	31	28	(a)
	11	26	(b)
	14	19	(c)
	65	105	(d)
AGE RANGE (YEARS)	25-25	12-40	(a)
	3-15	18-35	(c)
	1-34	7-64.5	(d)
CYSTIC FIBROSIS	31	2	(a)
PROTEIN PRESENT	10	2	(b)
(No. SAMPLES)	6	4	(c)
	63	9	(d)

(a) Wilson G. et al - Pediatr Res 11 (1977) 986

(b) Tuly G. et al - Pediatr Res 13 (1979) 1078

(c) Our results

(d) Wilson G. and Fudenberg - Pediatr Res 9 (1975) 635.

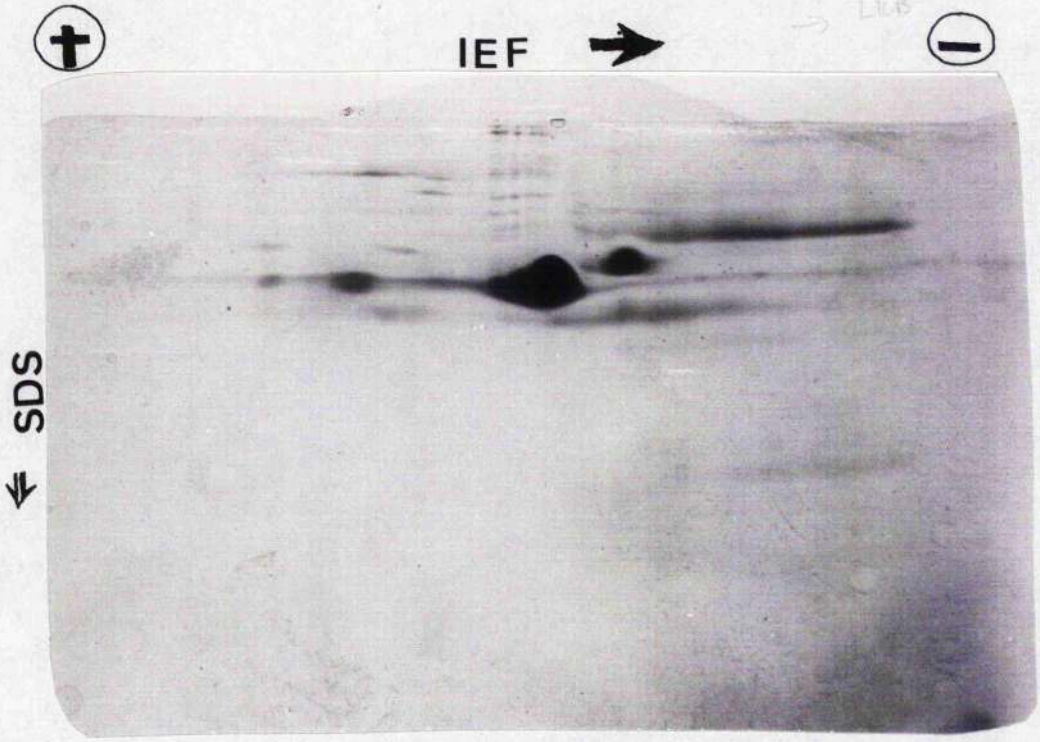


Figure 25 Two dimensional gel electrophoresis results of partially denatured serum from normal control subject. The proteins were separated in 15% gel in the second dimension.

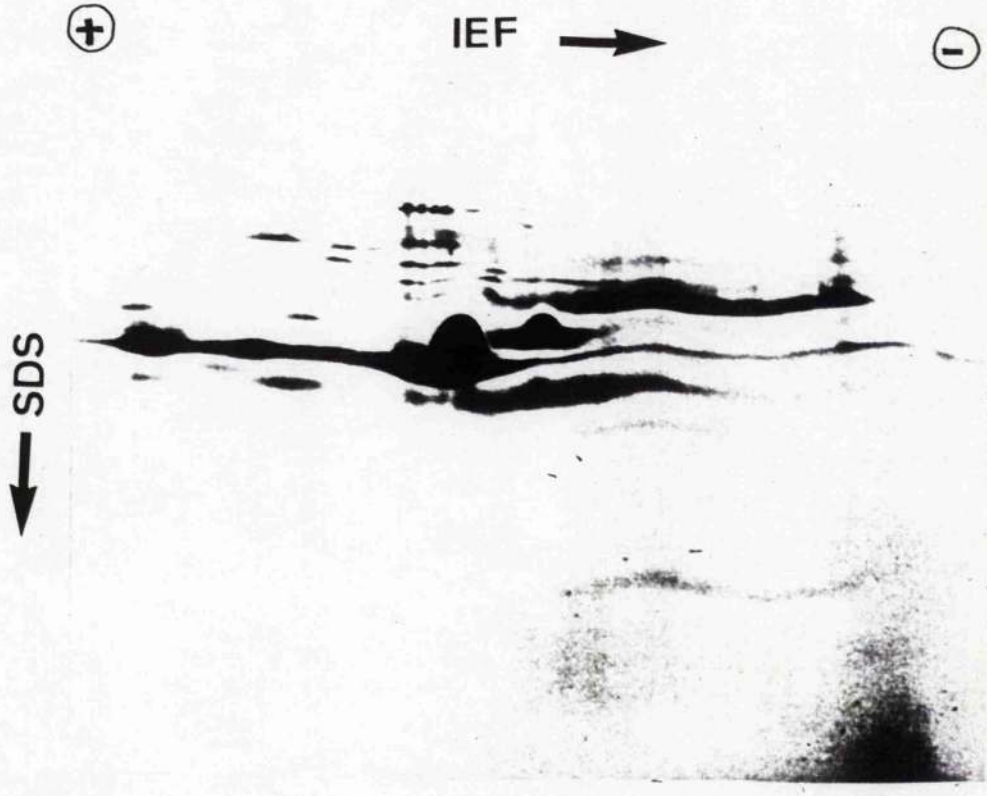


Figure 26 Two dimensional gel electrophoresis results of partially denatured serum from a cystic fibrosis patient.

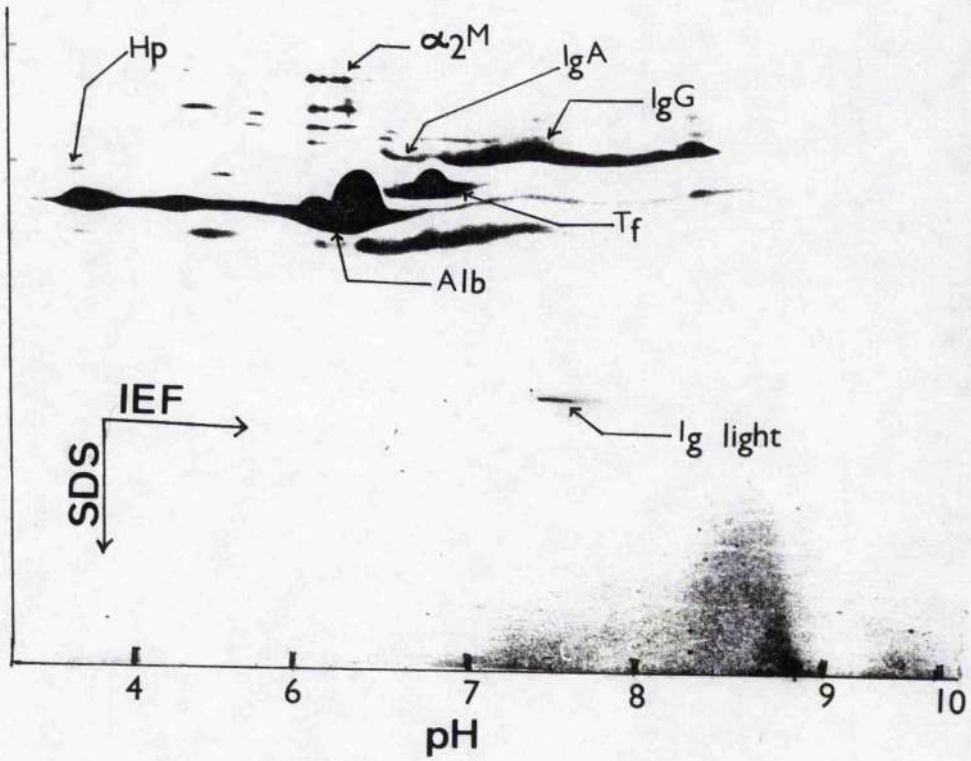


Figure 27 Mapping of the gel shown in Figure 26. The proteins were named according to Manabe et al. - J. Bioch 85 (1979) 649. Hp = Haptoglobin; Alb - Albumin; α_2M - alpha-2-macroglobulin; Tf - Transferrin.

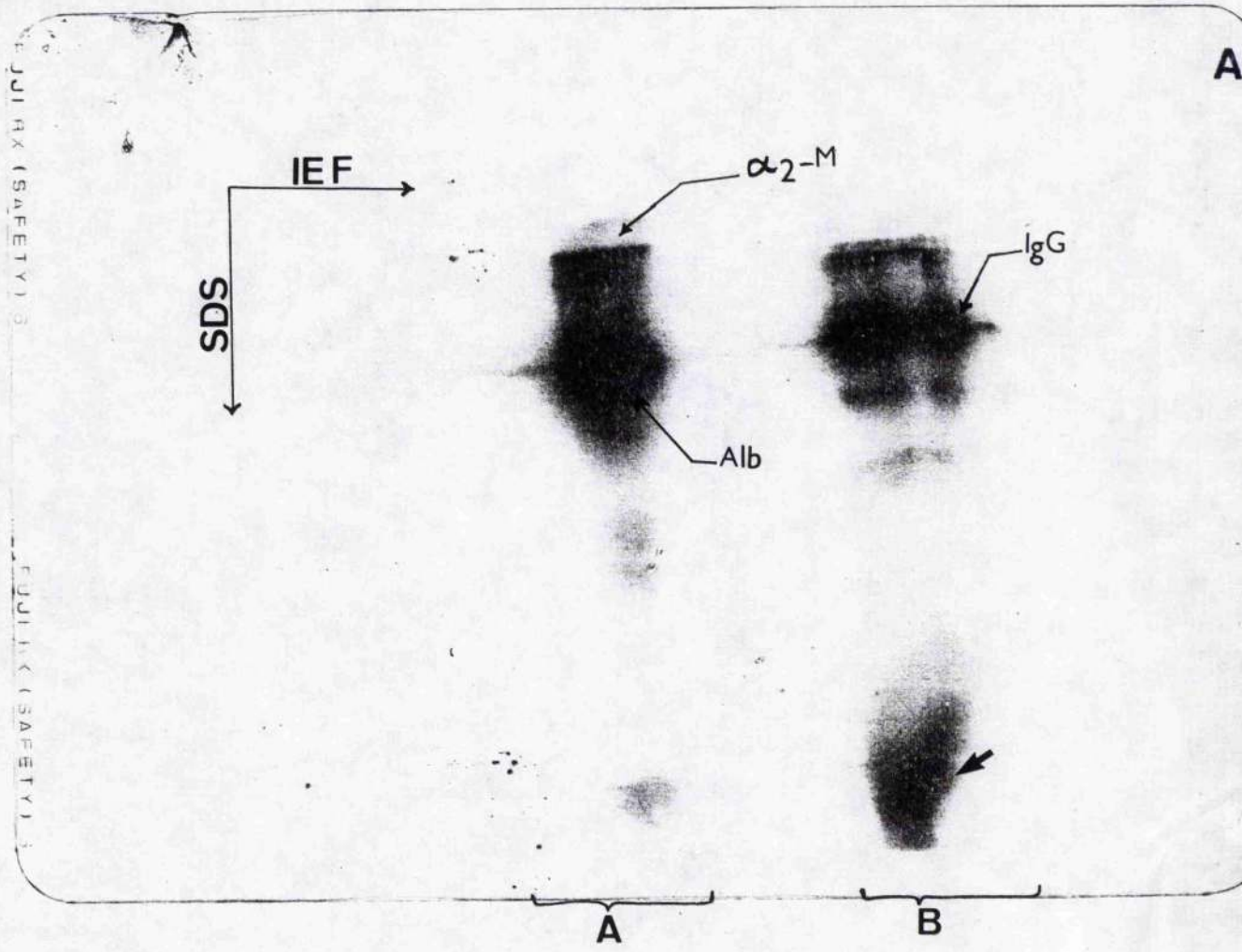


Figure 28 Autoradiography results of selectively labelled proteins. The proteins that focused between pH 4.5 - 6 (A) and pH 8 - 9 (B) were labelled.

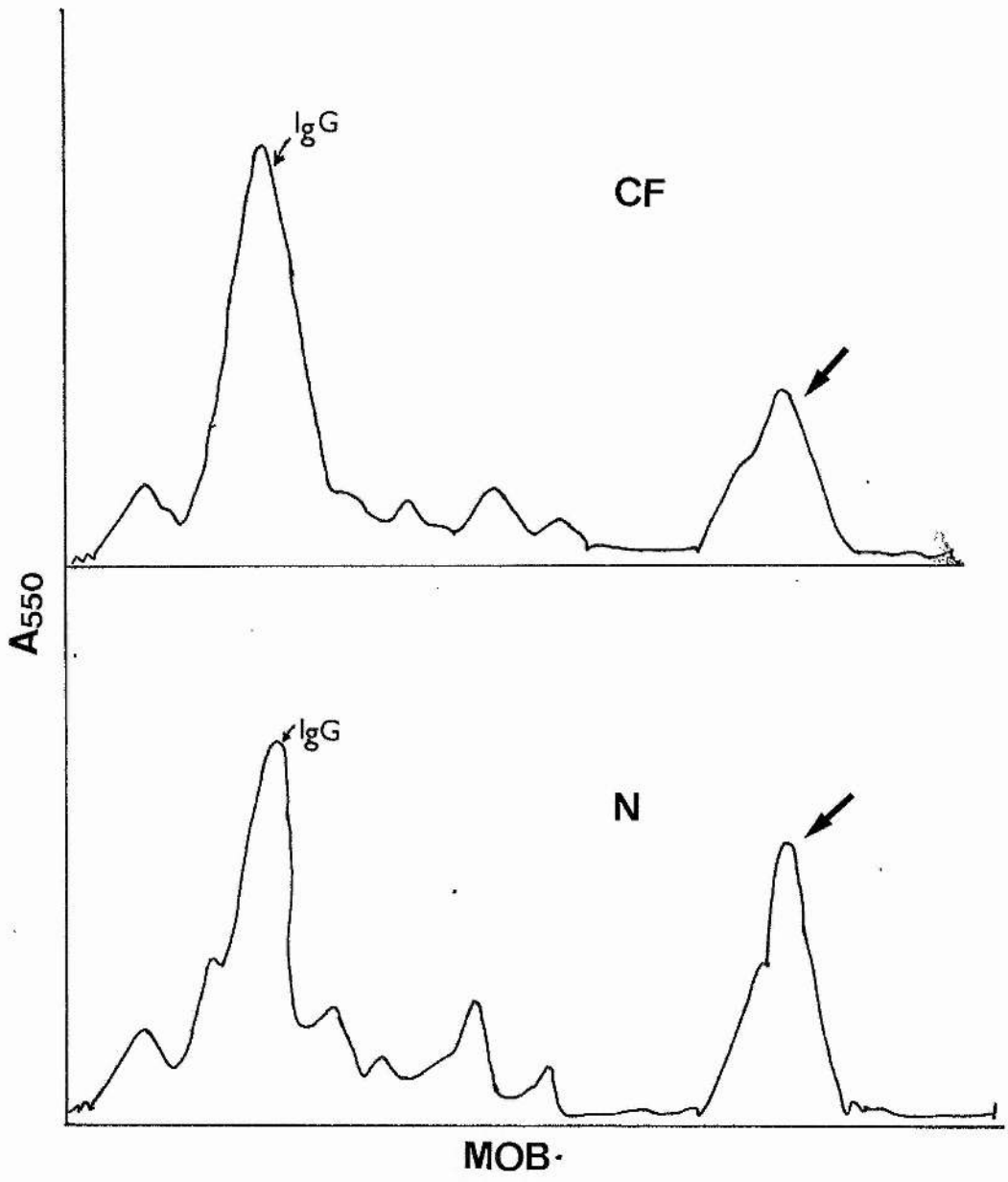


Figure 29 Densitometry recordings of SDS polyacrylamide gel electrophoretic peptide patterns of serum proteins that focused between pH 8 - 9. The low molecular weight peptides are indicated by arrows.

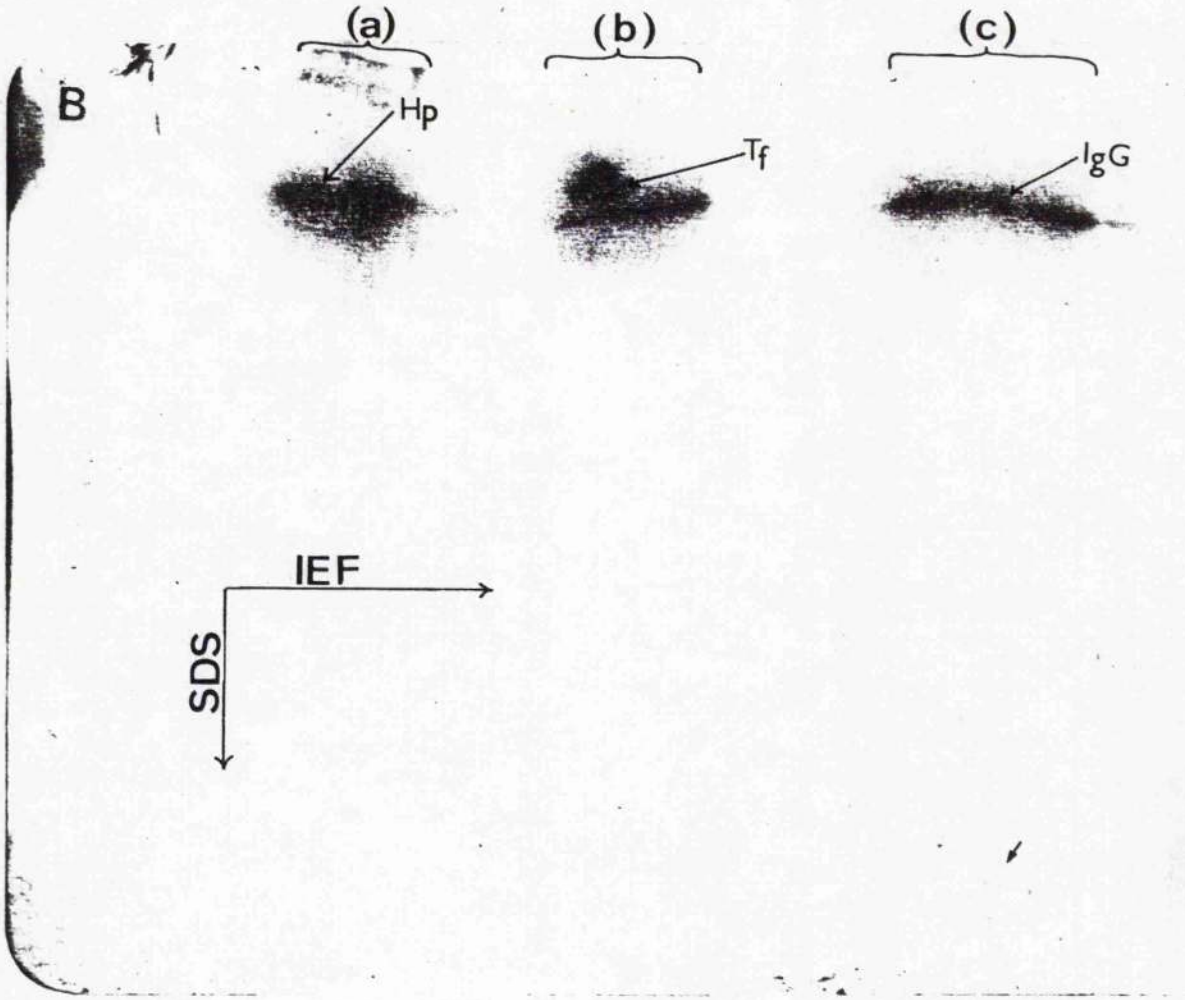


Figure 30 Selective labelling of Haptoglobin (Hp); Transferrin (Tf) and Immunoglobulin G (IgG).

7.1 Isoelectric focusing of serum samples

Typical results of isoelectric focusing of serum samples from normal control subjects and cystic fibrosis patients in a pH2.5-10 gradient are presented in figures 21-23. The results show great improvements in bands resolution when small sample sizes (5-10μl) were run. More than 40 protein bands could be resolved in this system. The patterns we obtained were similar to those obtained by Wilson et al (1977) using the same procedure. It is evident from the figures that the resolution of individual bands decreased with increases in sample sizes and since the sample application pads were not removed during the run, trailing occurred.

The results of screening for the cystic fibrosis factor by isoelectric focusing of sera from patients and normal control subjects are presented in figures 21 - 23. To ascertain that there were no false negative results, volumes of 15-50μl of each sample were applied. Fourteen cystic fibrosis and 19 normal control samples were screened for the CF factor. Figure 23 shows the enlargement of part of the most cathodal region of the polyacrylamide gels. Arrows indicate the positions of the double protein bands of the cystic fibrosis factor. The factor focused at pH8.5, a value that agrees with the pI's of 8.4 and 8.5 reported by Nevin (1978) and Wilson et al (1977) respectively. The pH gradient curve (figure 24 curve A) was estimated with a surface electrode at 4^{o}C. Of the 14 samples from cystic fibrosis patients of ages 3-15 years and 19 from normal control subjects screened for the cystic fibrosis factor, six of the patients and four of the normal control subjects samples showed the extra protein bands at pH8.5 (Table VI). Samples that contained slight haemolysis seemed to have some interference of the banding pattern at

the most cathodal end.

The combined technique of isoelectric focusing and SDS gel electrophoresis under partially dissociating conditions was adopted here to analyse serum proteins from normal control subjects and cystic fibrosis patients. The first dimension isoelectric focusing was performed in a pH2.5-10 gradient on an LKB multiphor by the method of Wilson et al (1977). The SDS gel electrophoresis, in the second dimension was performed in a Laemmli discontinuous gel system as described in methods. The optimal volume of serum used in this procedure was 10μl. Typical two dimensional serum protein patterns obtained are shown in figures 25-27. Samples from seven control subjects and five patients were analysed and compared. There are no consistent distinguishing differences between patients and control samples. Figure 27 shows the mapping of protein bands obtained with major bands named according to Manabe et al (1979).

We searched for the cystic fibrosis protein by radiolabelling the proteins that focused between pH8.0-9.0. To achieve this without altering the pI's of proteins, labelling was performed after electrofocusing. The chloramine-T method was used to iodinate proteins with ^{125}I as described in methods. Figure 28 shows the typical radiographs obtained after labelling selected regions of the focused gel.. Low molecular weight peptides labelled (indicated by arrows in Figures 28 and 29) possibly contained the cystic fibrosis protein. Scanning of labelled bands (Figure 29) in an attempt to quantitate them could not show any distinguishing differences between control and cystic fibrosis samples. Proteins that focused between pH5 and pH6 were also labelled. The region contained α_2M which is believed to be abnormal in cystic fibrosis. No differences could be observed between cystic fibrosis and normal samples.

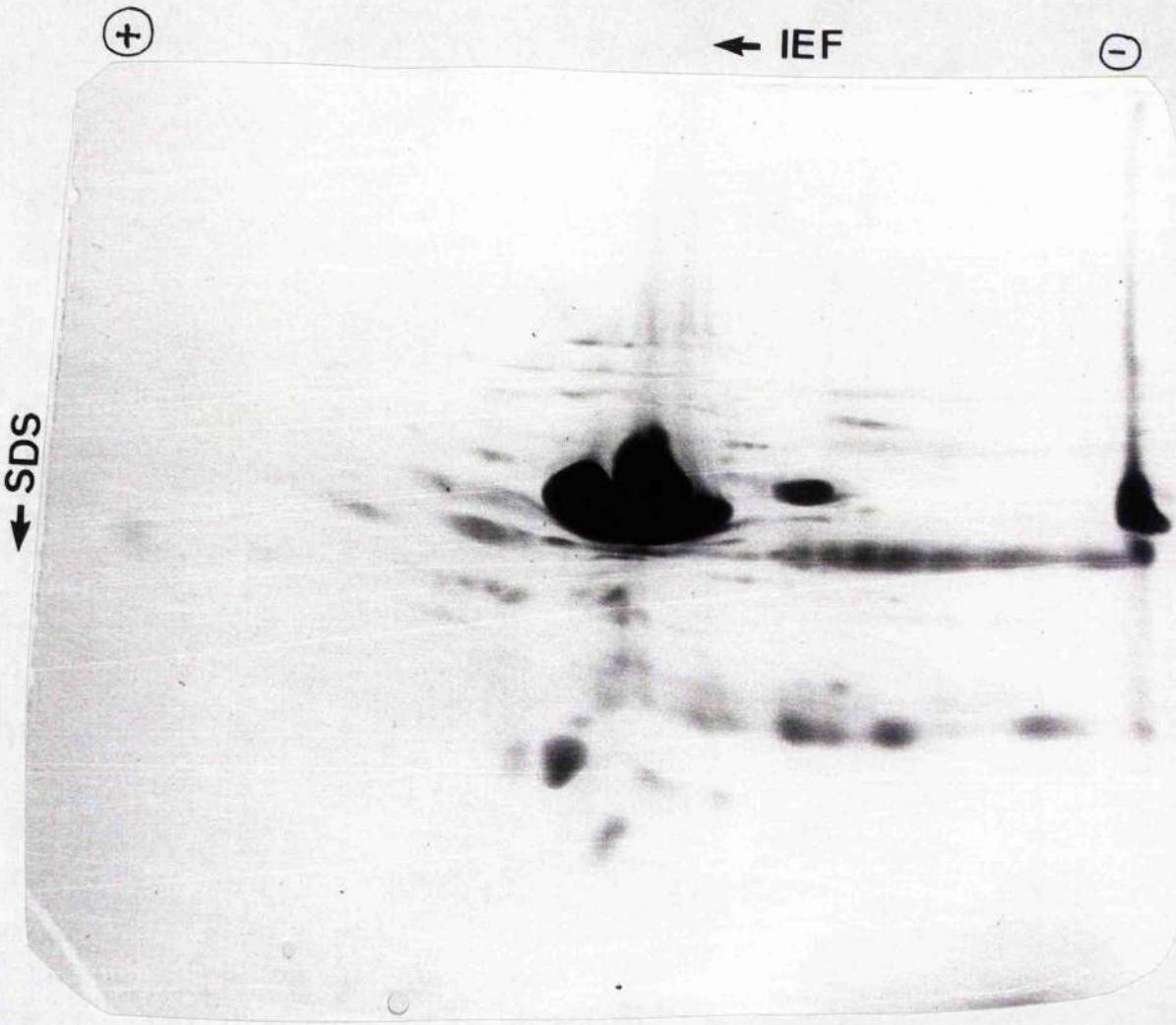


Figure 31 Two dimensional gel electrophoresis of serum from a control subject. 15μl of sample were denatured in SDS and mercaptoethanol and then analysed in 7-24% linear gradient gel.

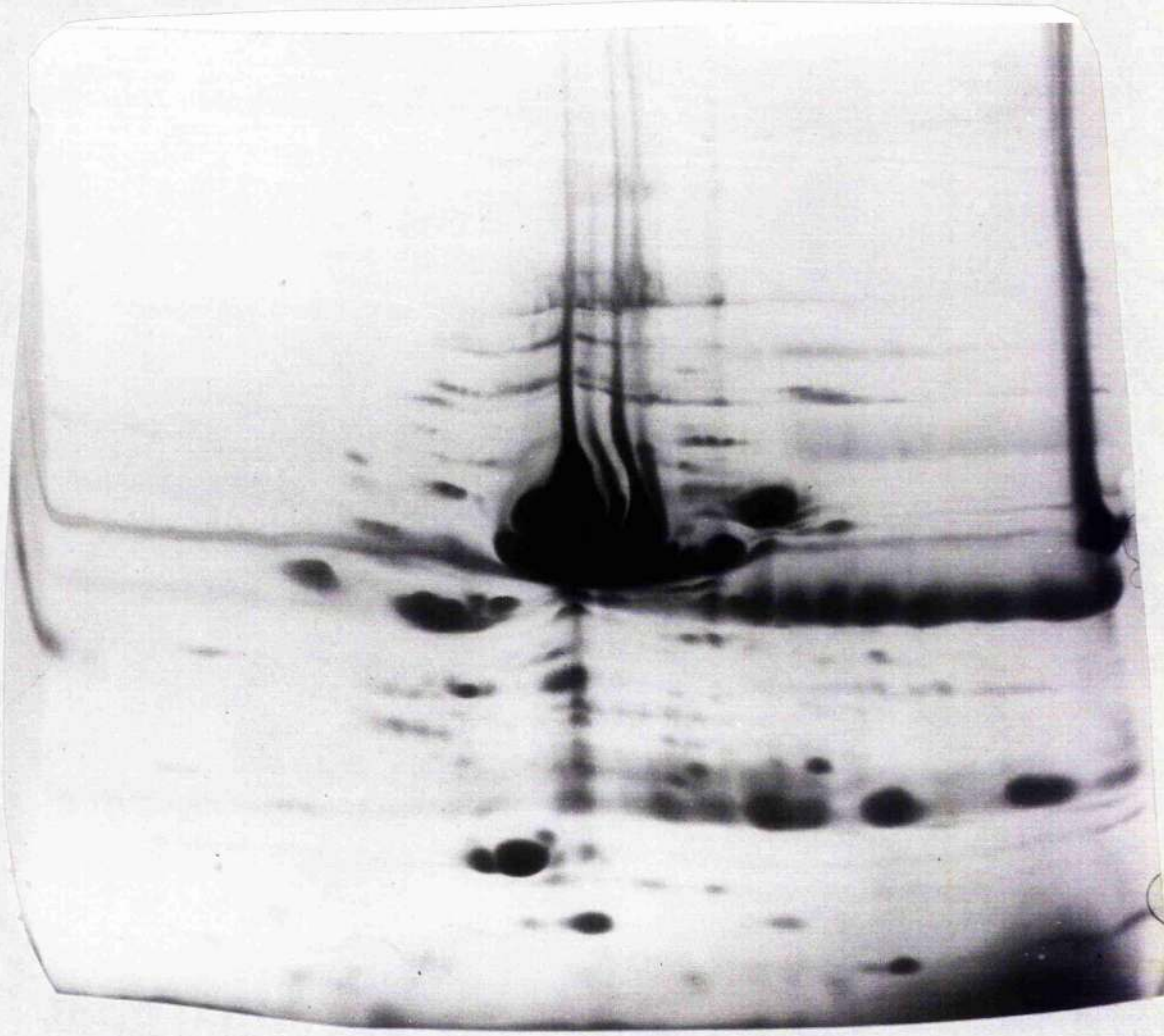


Figure 32 Same as in Figure 31 except that 20μl of serum were used.

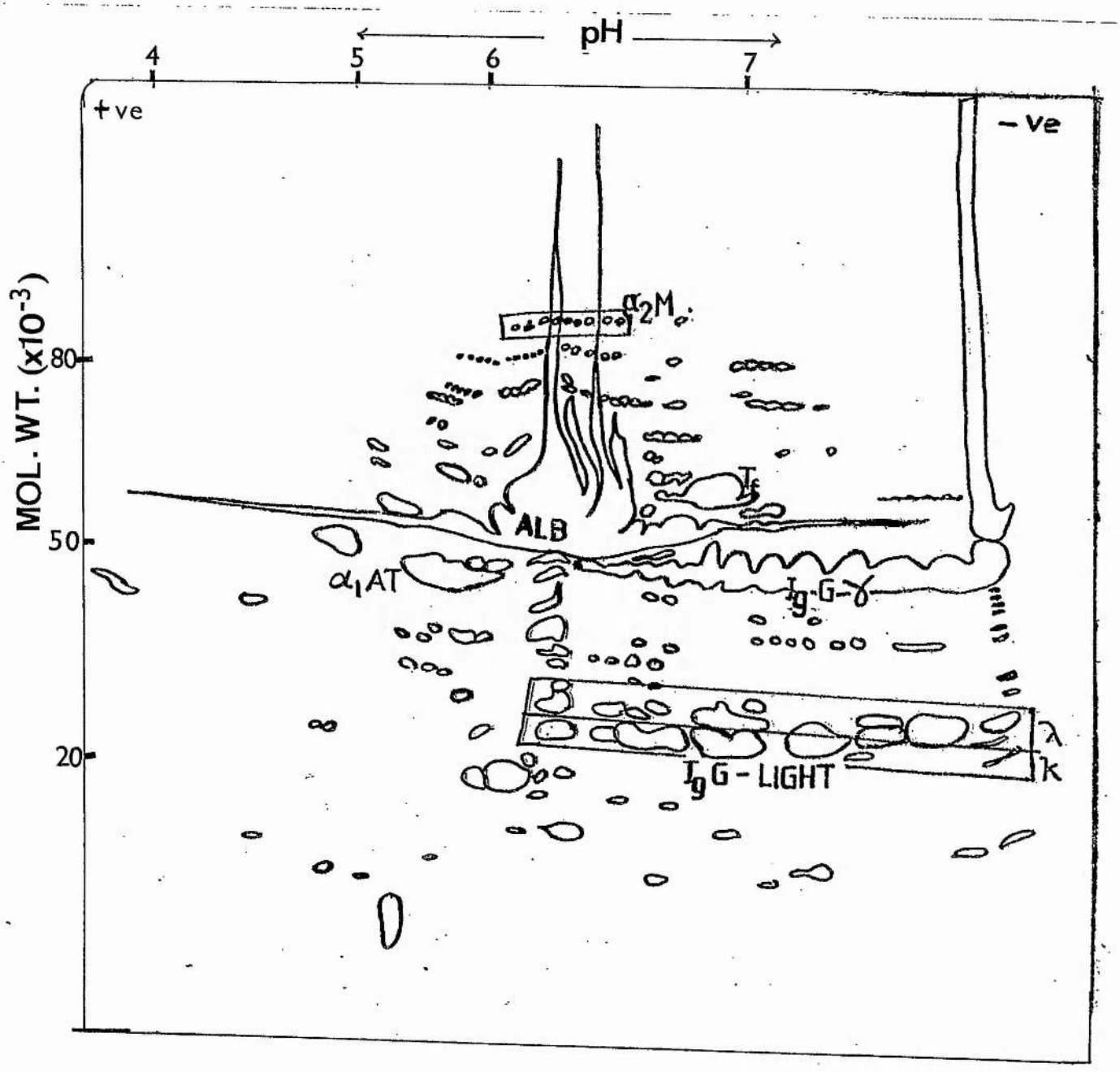


Figure 33 Diagrammatic representation of the gel shown in Figure 32. The major proteins bands were named according to Anderson and Anderson (1977).

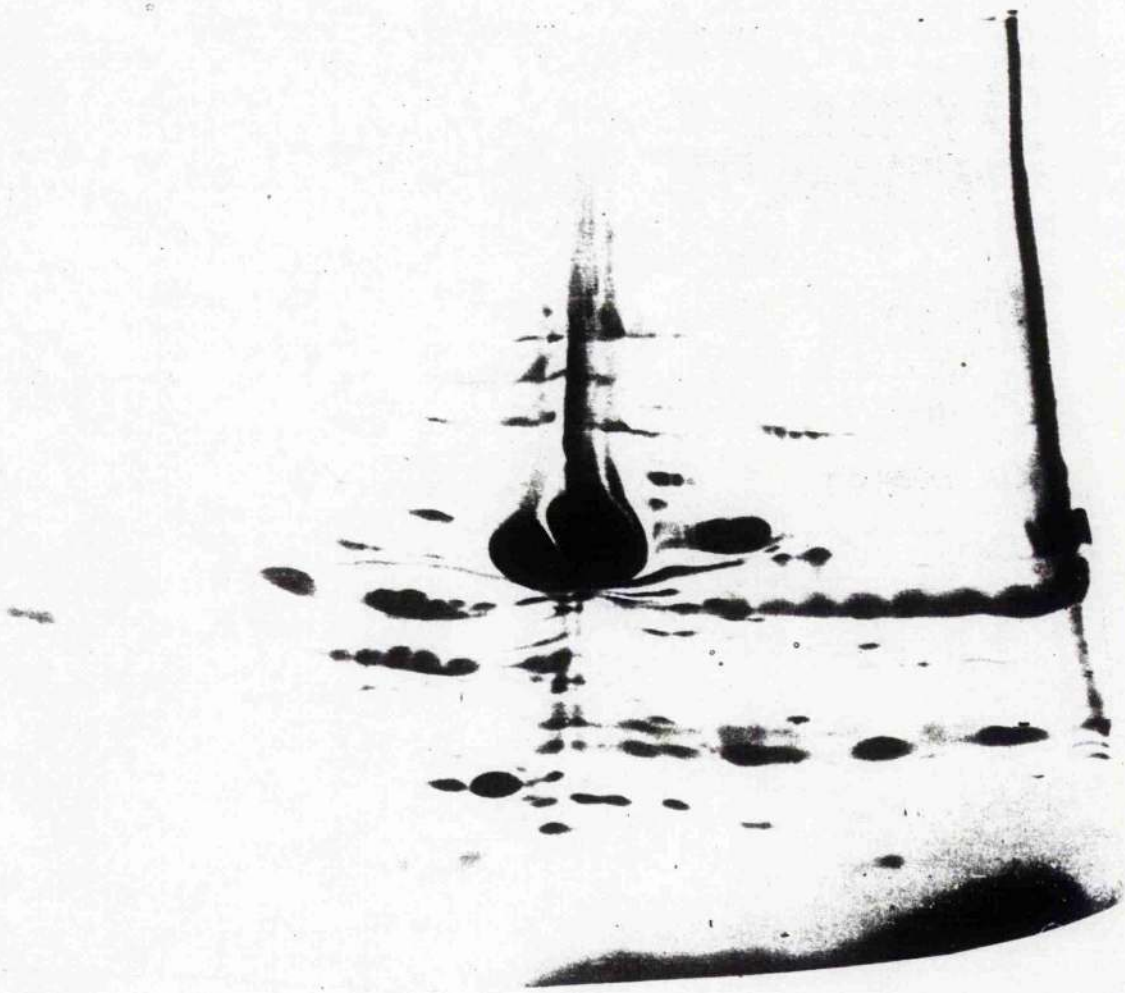


Figure 34 Two dimensional gel electrophoresis of 20μl of serum from cystic fibrosis patient.

Figure 35 A pattern of proteins on a linear gradient gel as in Figure 31 except that the serum proteins were from a cystic fibrosis patient and that they were visualised by silver staining.

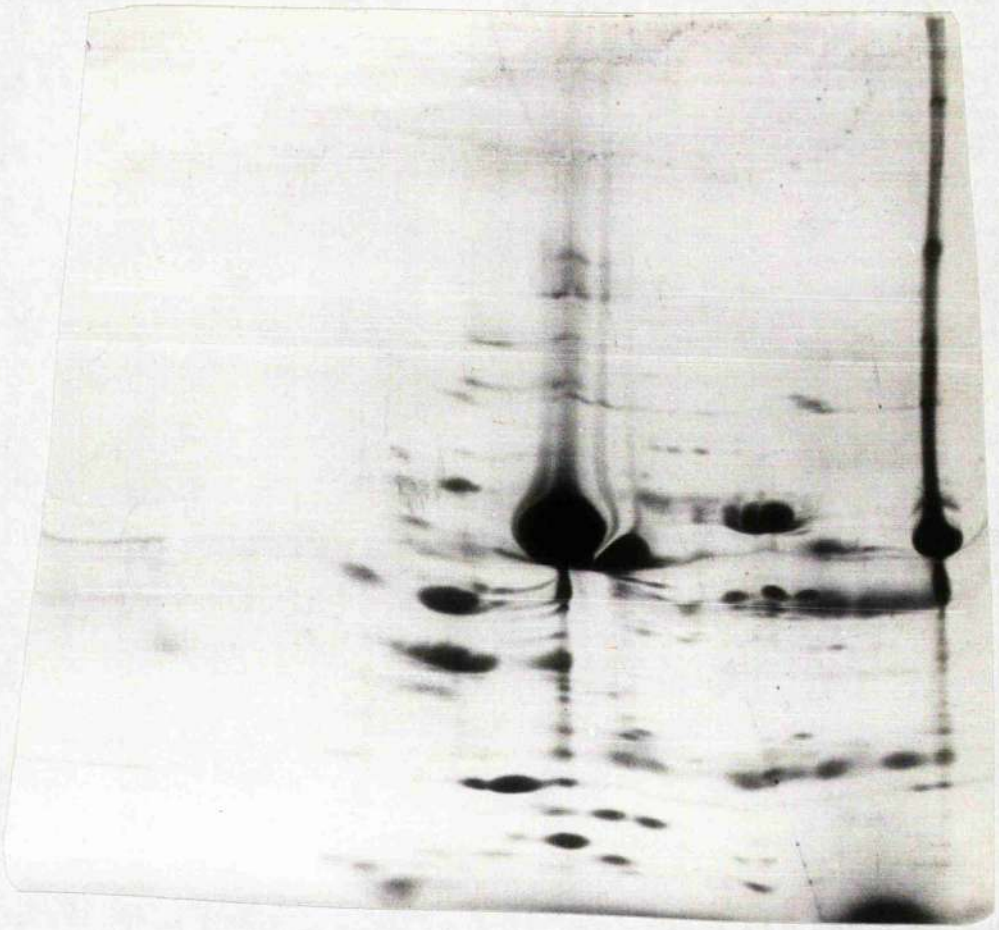


Figure 36 Two dimensional gel electrophoresis of plasma proteins. The sample was 20μl of plasma from a multiple sclerosis patient.

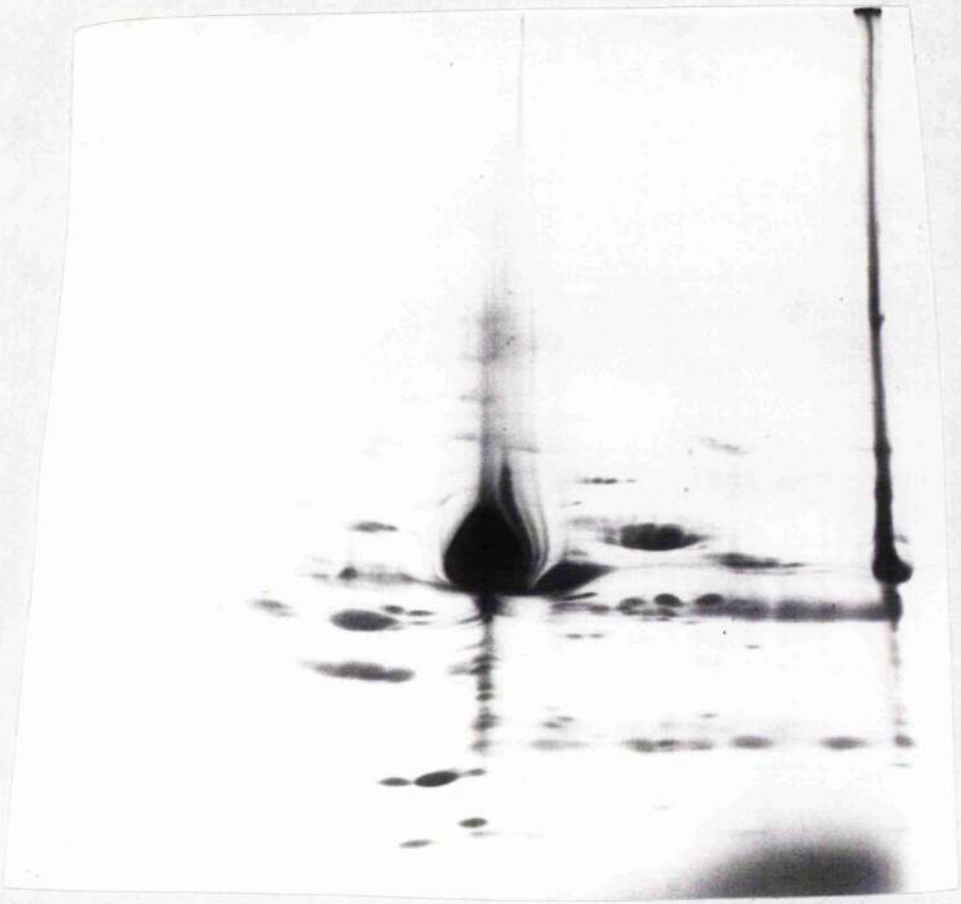


Figure 37 Two dimensional gel electrophoresis of plasma proteins from a control subject.

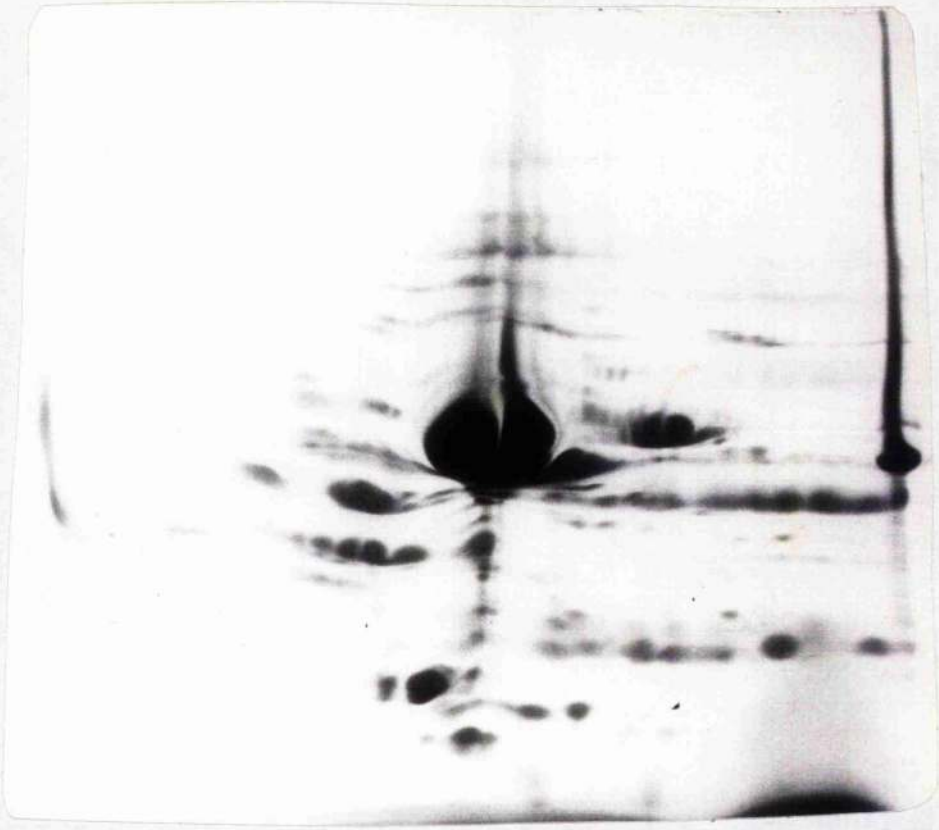


Figure 38 Two dimensional gel electrophoresis of serum proteins from a multiple sclerosis patient.

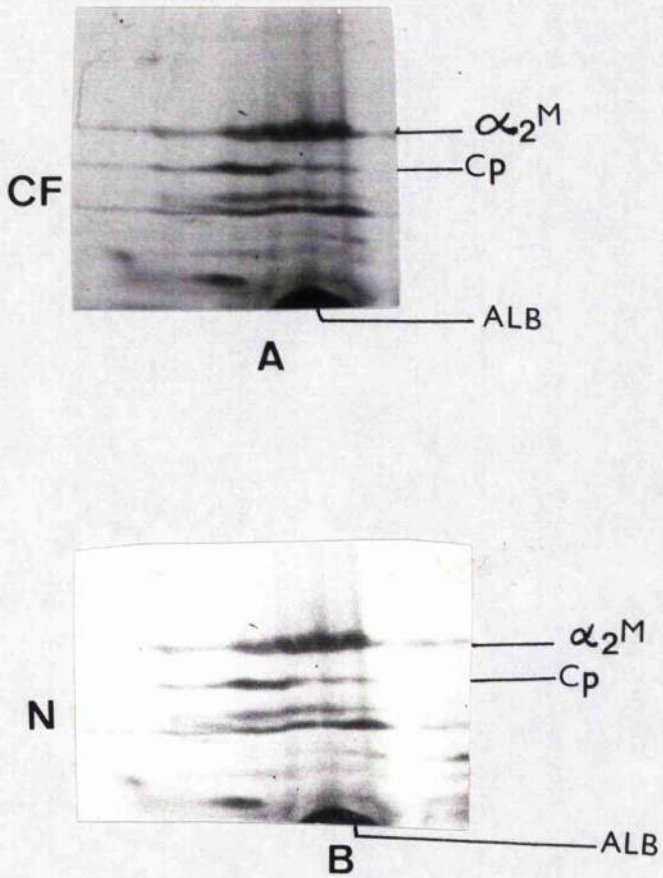


Figure 39 Sections of two dimensional gels showing α_2M (alpha-2-macroglobulin) and Cp (ceruloplasmin) of cystic fibrosis patients and normal subjects.

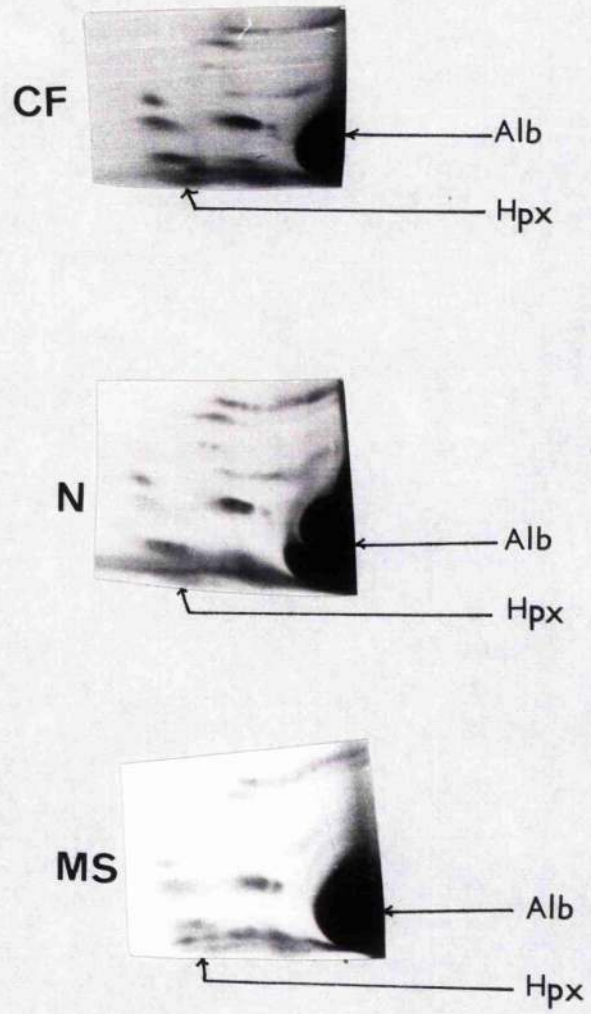


Figure 40 Sections of two dimensional gels comparing samples from cystic fibrosis and multiple sclerosis patients and normal subjects. Hpx, Haemopexin.

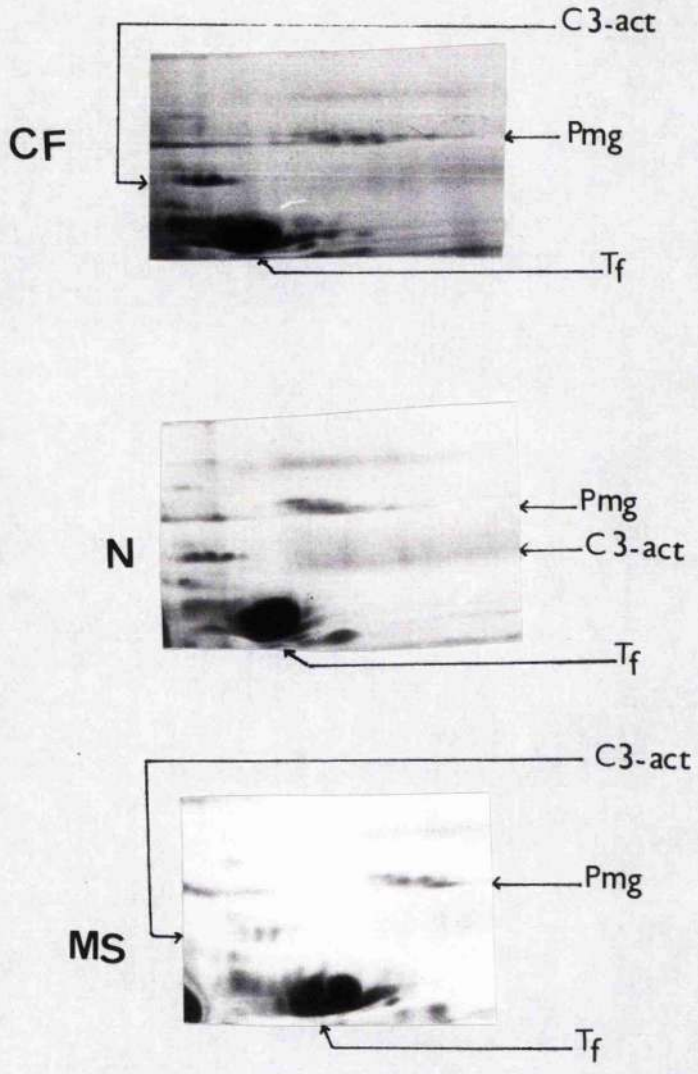


Figure 41 Sections of two dimensional gels showing C3 act, C3 activator; Pmg, plasminogen Tf, Transferrin.

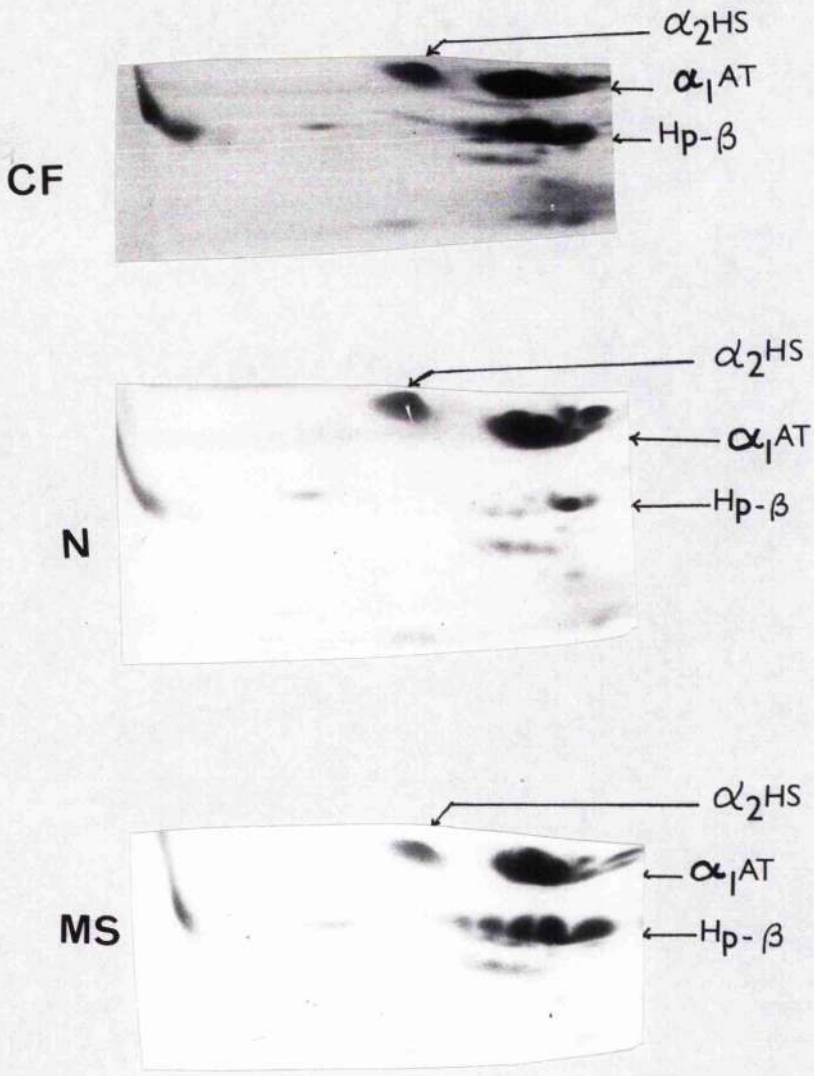


Figure 42 Sections of two dimensional gels showing variants of α_1AT, alpha-1-antitrypsin; and Hpβ, haptoglobin β- chains. α_2HS glycoprotein is also labelled.

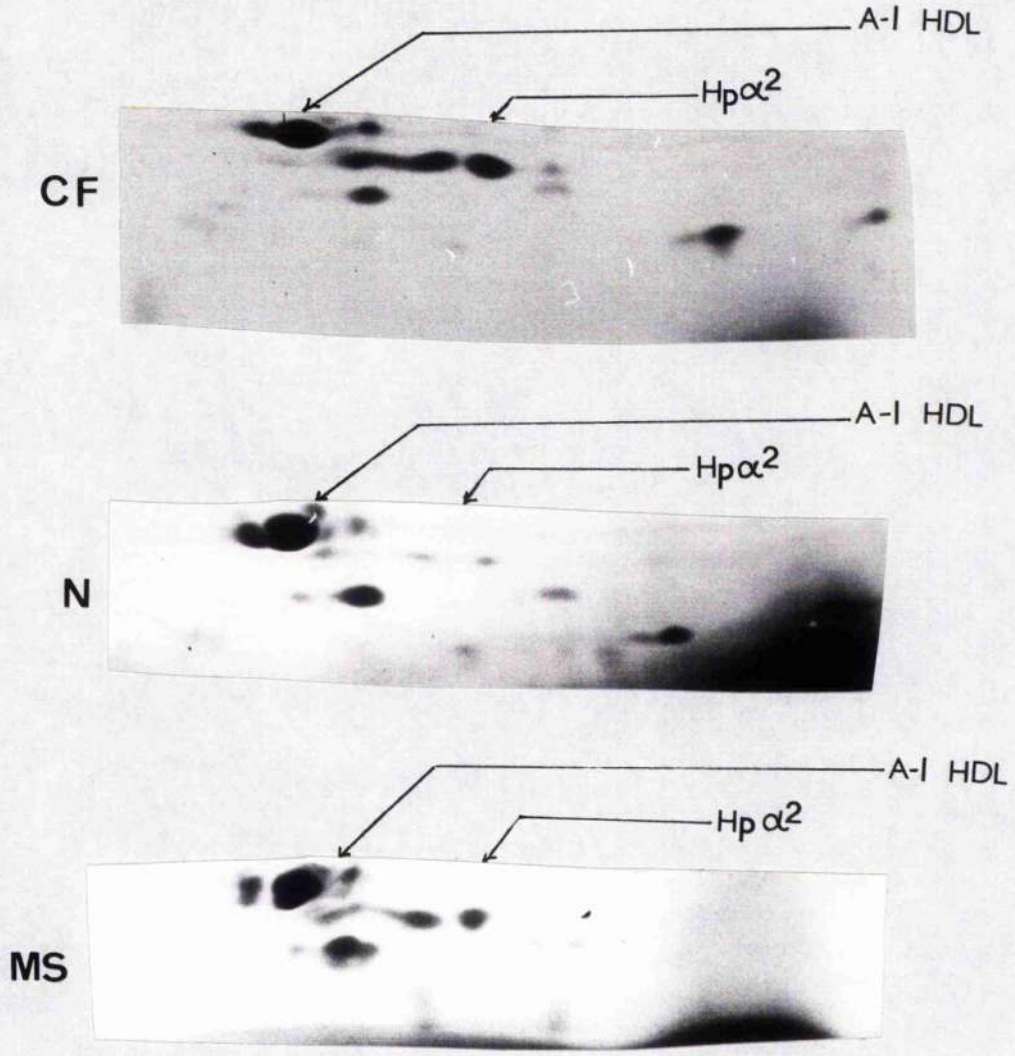


Figure 43 Sections of two dimensional gels showing variants of haptoglobin α-2 chains (Hp α-2). A-1 HDL = apo A-1 lipoprotein.

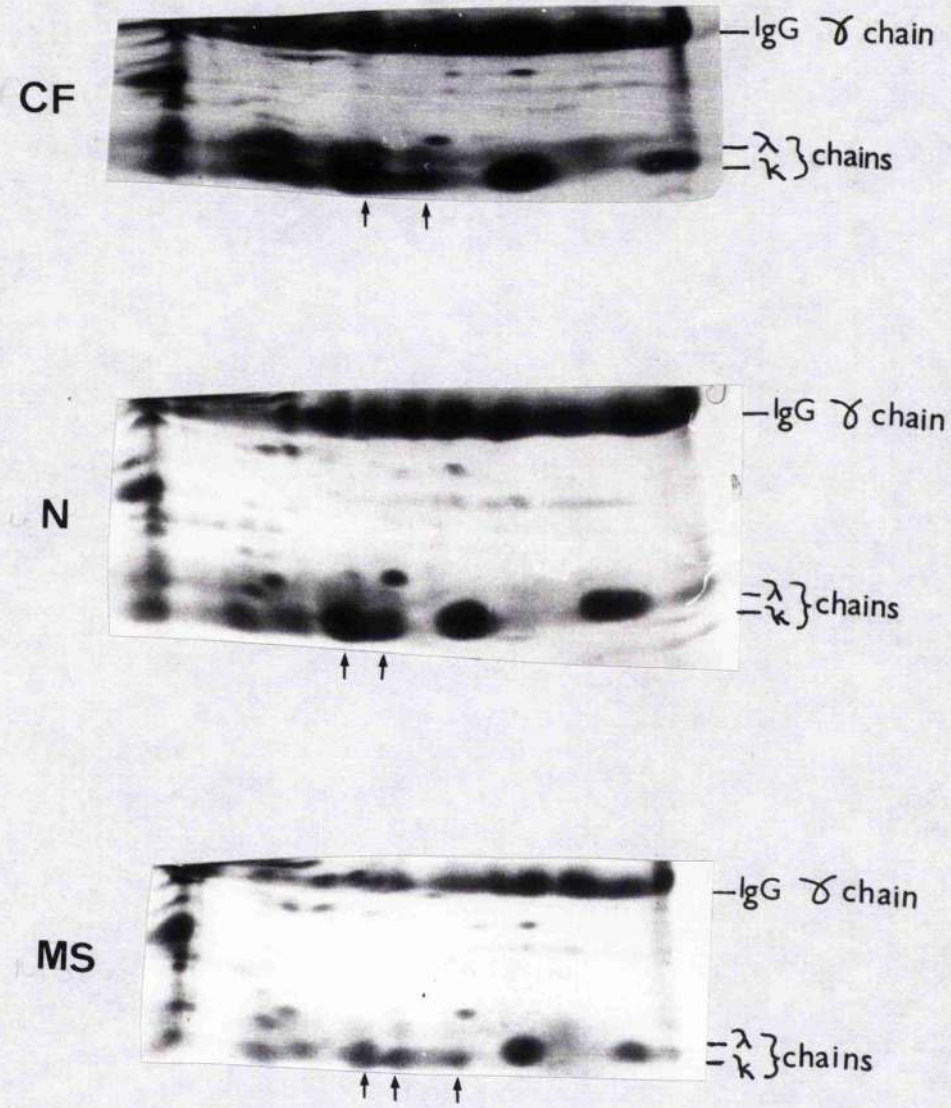


Figure 44 Sections of two dimensional gels showing immunoglobulin γ, λ and κ chains. Extra peptide is evident in multiple sclerosis gel (arrows).

7.2 Two dimensional gel electrophoresis under complete dissociating conditions

Two dimensional gel electrophoresis under complete dissociating conditions involved (I) Heating 20μl of sample with 40μl of 2% (w/v) SDS/5% (v/v) 2-mercaptoethanol/10% (w/v) glycerol. (II) Isoelectric focusing in 9M urea/2% (w/v) NP-40 and (III) SDS gel electrophoresis in 7-24% gradient gel. The procedure is able to separate individual gene products, thus makes it a suitable method for analysing genetic variants. We used the method to analyse serum or plasma samples from cystic fibrosis and multiple sclerosis patients and control subjects. Typical results obtained are shown in Figures 31-38. Overloading of albumin (Figures 32-38) was necessary to visualise the less abundant peptides. Over 200 peptides were visualised in Figures 32-38. Attempts to improve detection by using the silver staining method of Merril et al (1979) were not very fruitful. Though the silver staining method was sensitive, the less abundant proteins faded within minutes of staining (Figure 35). The other problem with this method was the high quantities of silver nitrate needed - an expensive material. Comparisons of protein patterns of samples from cystic fibrosis and multiple sclerosis patients and control subjects were unable to show any differences. Control subjects with other neurological diseases were also included. Genetic variants were observed but these were not specific for either cystic fibrosis or multiple sclerosis. Figures 39-44 show sections of the two dimensional gels with some major proteins named according to Anderson and Anderson (1977). Figure 39 shows identical α_2M peptide patterns in cystic fibrosis and normal control samples. Variants of albumin (Figures 31-38), transferrin (Figure 41), haptoglobin β (Figure 42) and haptoglobin α (Figure 43) chains were obtained but were not related

to the diseases under investigation. Of particular interest was the extra peptide in immunoglobulin κ chains in samples from multiple sclerosis patients (Figure 44). This peptide was also present in samples of control subjects with other neurological diseases.

7.3 Analyses of α_2-macroglobulin preparations from cystic fibrosis and multiple sclerosis patients.

Three multiple sclerosis, four cystic fibrosis and six normal control α_2M samples were prepared from serum samples of polyethylene glycol (PEG) precipitation and chromatography on Blue Sepharose Cl-6B in 0.05M Tris-HCl buffer pH8.0 as described in methods. Typical elution patterns obtained are shown in Figure 45. The first 4 tubes, (Figure 45 - curve A) showing absorbance at 280nm contained α_2M with minimal contamination. Figure 46 shows the electrophoregrams of the first seven fractions containing some protein. Later fractions (tubes 24 and 30) are also shown to portray the contaminations eluting in these fractions.

Incubation of α_2M samples at 20°C with SDS sample buffer consisting of 2% (w/v) SDS/5% (v/v) 2-mercaptoethanol/10% (w/v) glycerol yielded a single protein band with an apparent molecular weight of about 180 000 when analysed by SDS gel electrophoresis (Figure 47B). The appearance of this band was coupled by the disappearance of a double band designated as S-α_2M and F-α_2M with apparent molecular weight of 725 000 (Figure 47A). Incubation of α_2M with SDS sample buffer at 95°C for 5 minutes produced two more protein bands with apparent molecular weights of 130 000 and 62 000 (Figure 47C). Less abundant protein bands of apparent molecular weights 110 000 and 87 000 were just visible (arrows in Figure 48) in CF and control samples but these were pronounced in all our MS samples (Figure 48). Heat

induced fragments of α_2M prepared from cystic fibrosis and normal control serum samples were qualitatively identical (Figure 48) at least with respect to the molecular weights of the products. None of the normal or CF samples tested deviated significantly from this pattern. Two dimensional electrophoresis (Figure 50) and immunoelectro focusing (Figure 51) of α_2M from CF and normals produced identical patterns. Immunoelectrofocusing of α_2M from sera of MS patients produced a different pattern (Figure 52).

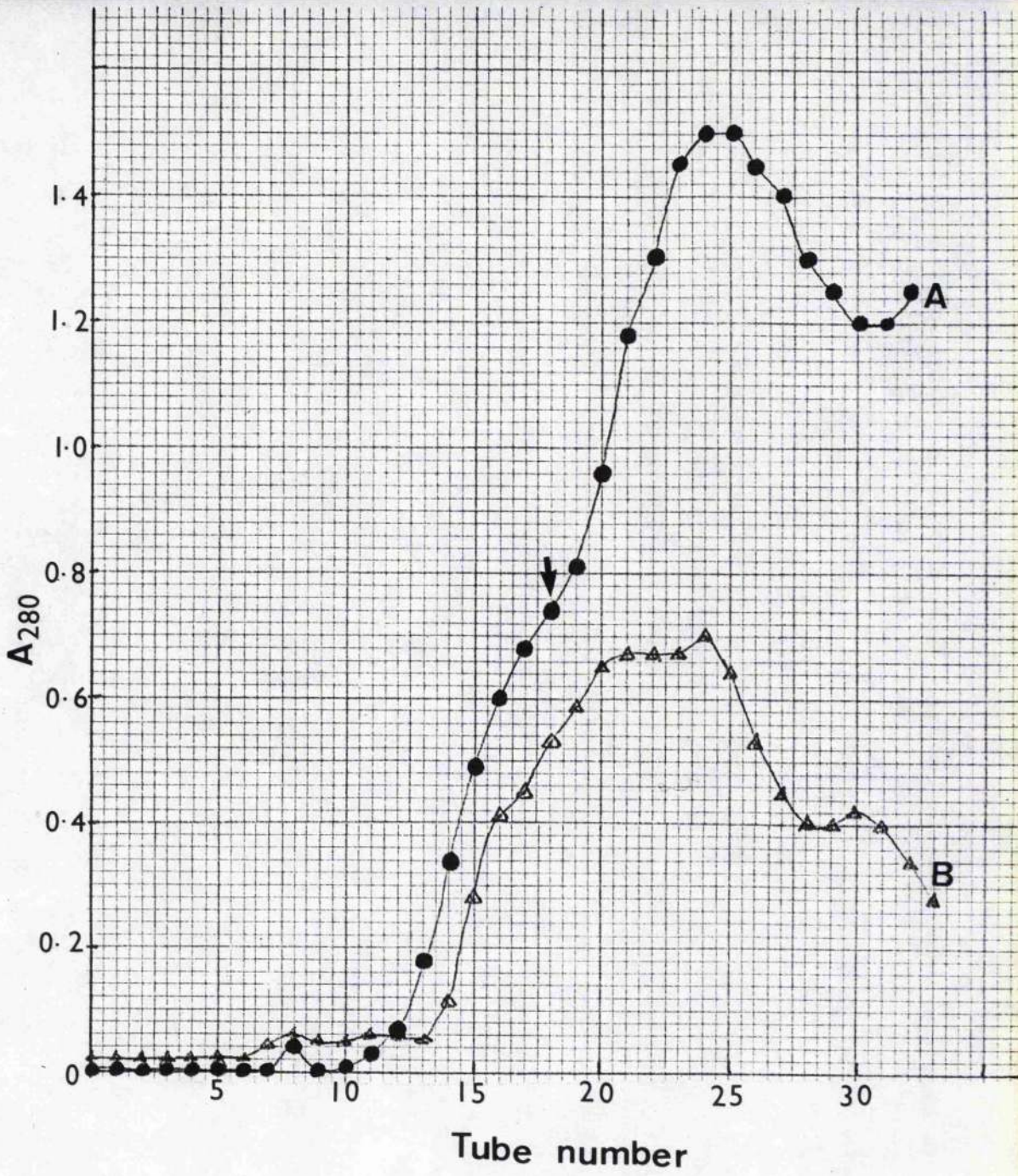


Figure 45 Elution diagram of fractions containing α_2M on sepharose Cl-6B. Curve A shows elution profile after PEG precipitation. Curve B shows profile for the fractionation of whole serum.

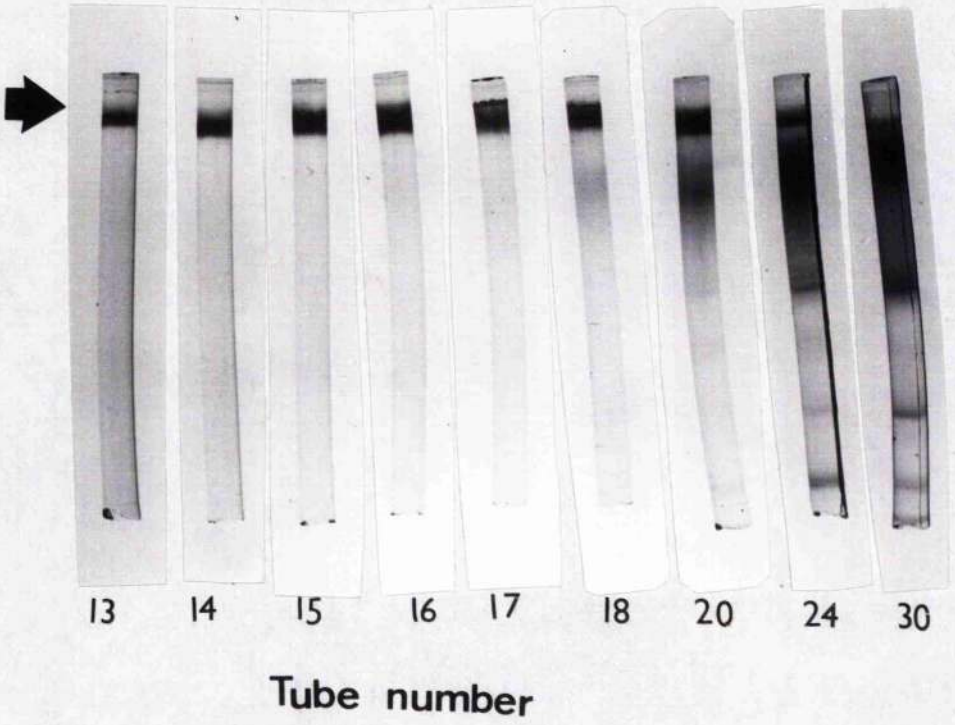


Figure 46 Gel electrophoresis of fractions that eluted from sepharose Cl-6B column in Figure 45A. Fractions that eluted early, tubes 13-17, contained α_2M with minimal contaminations.

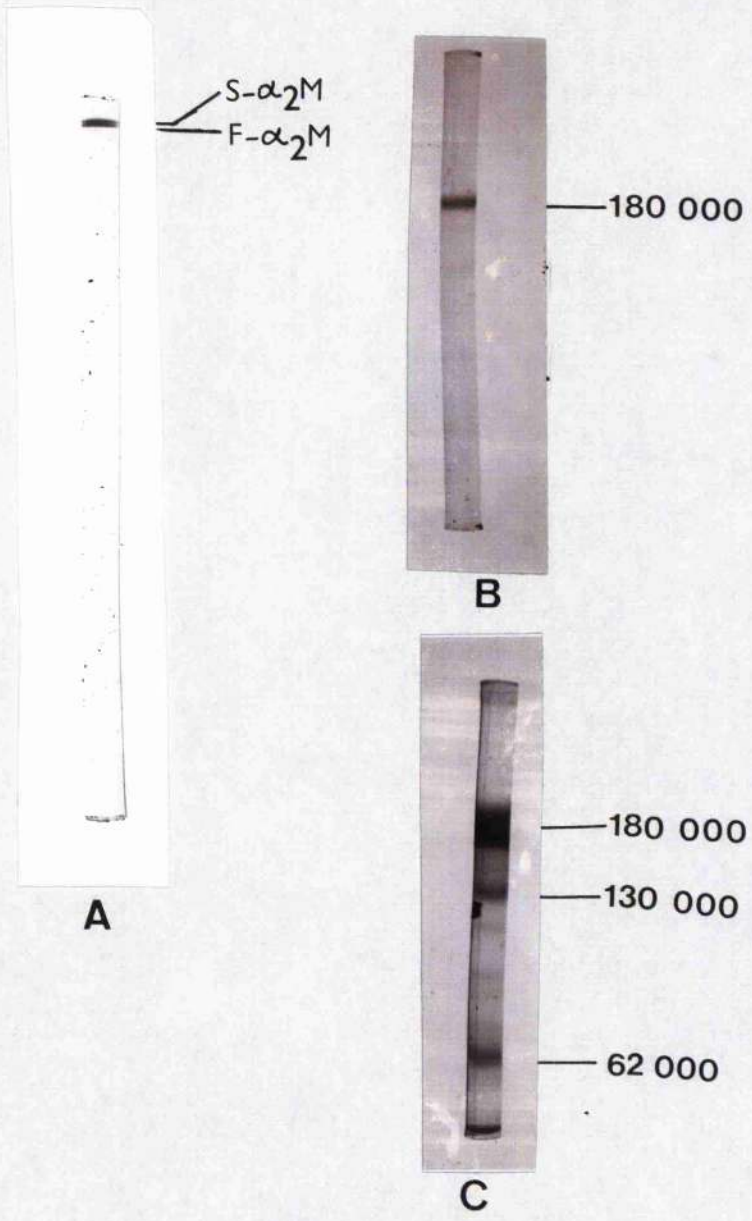


Figure 47 SDS gel electrophoresis of (A) native α_2M (B) α_2M denatured in SDS sample buffer at room temperature and (C) heat fragments of α_2M. Native α_2M was incubated with SDS sample buffer and heated at 95°C for 5 minutes.

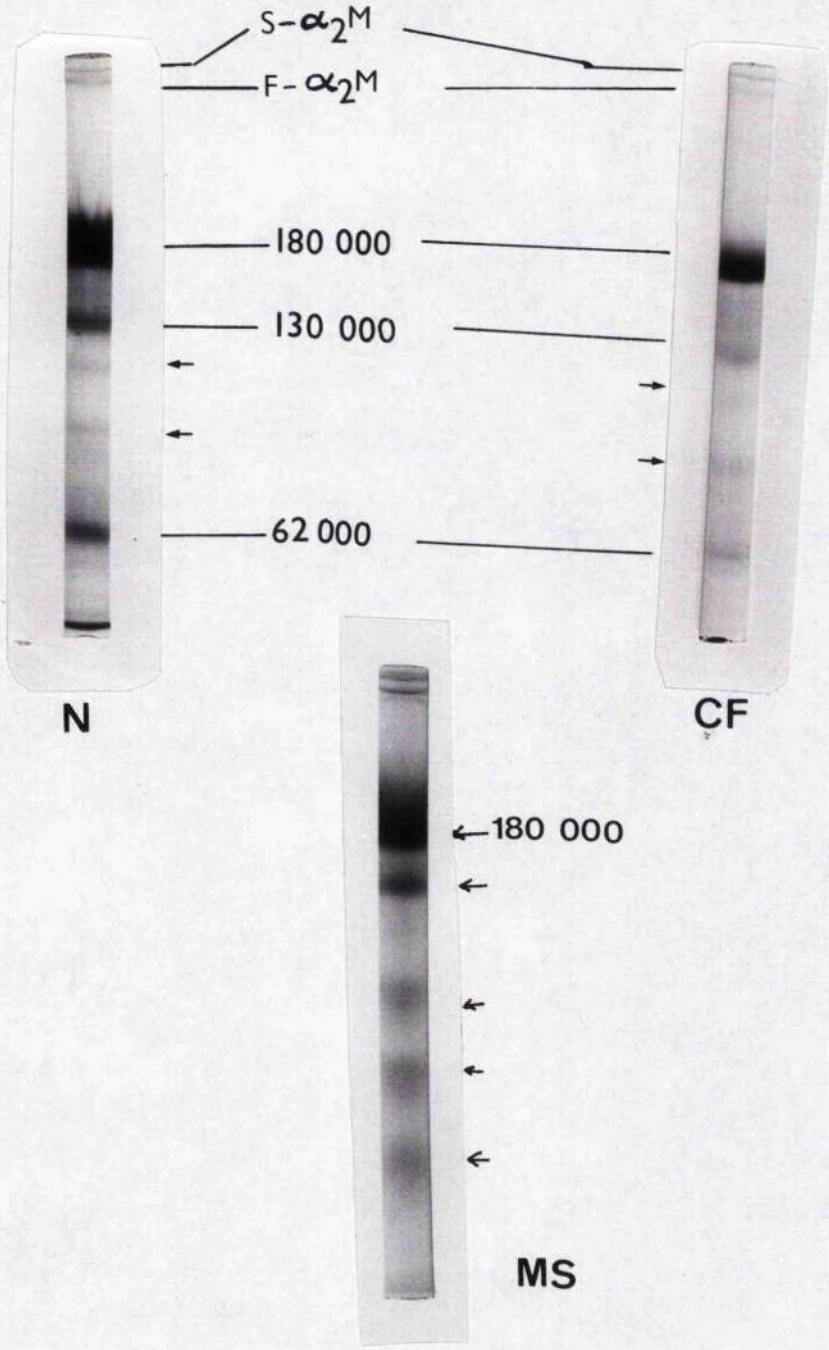


Figure 48 SDS gel electrophoresis of heat fragments of α_2M from multiple sclerosis and cystic fibrosis patients and normal control subject.

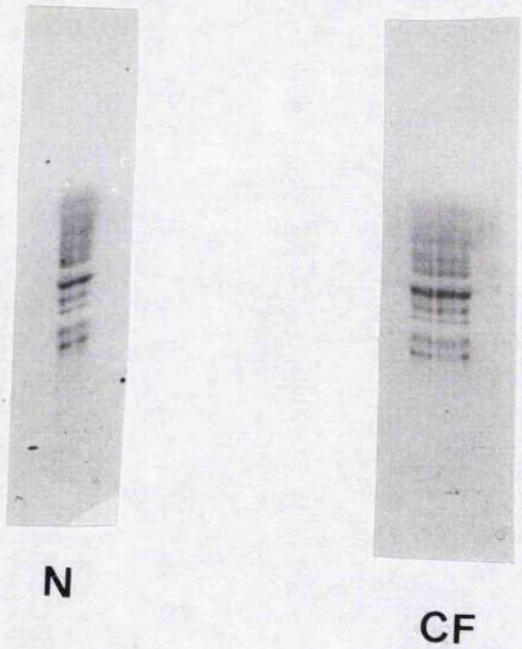


Figure 49 Isoelectric focusing of α_2M fractions prepared from sera of cystic fibrosis patient and normal control subject.

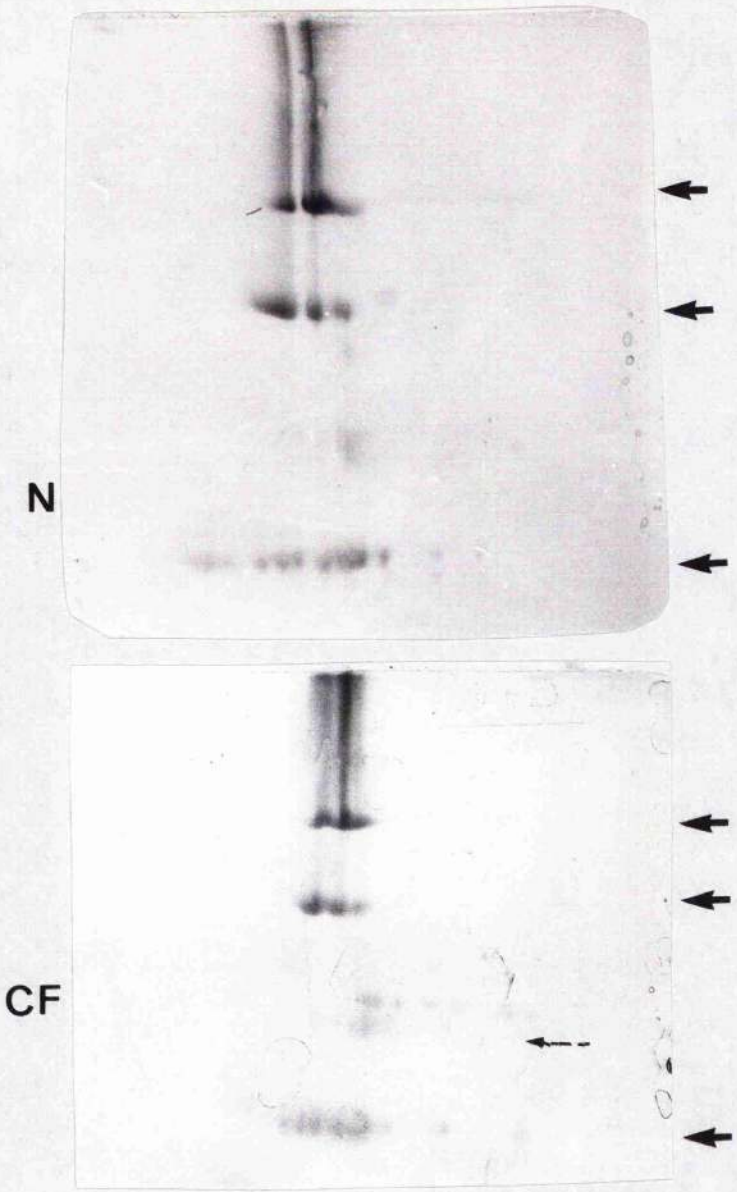


Figure 50 Two dimensional gels of α_2M fractions from normal control subject and cystic fibrosis patient.

Figure 51 Immunoelectrofocusing of α_2M from cystic fibrosis patient.

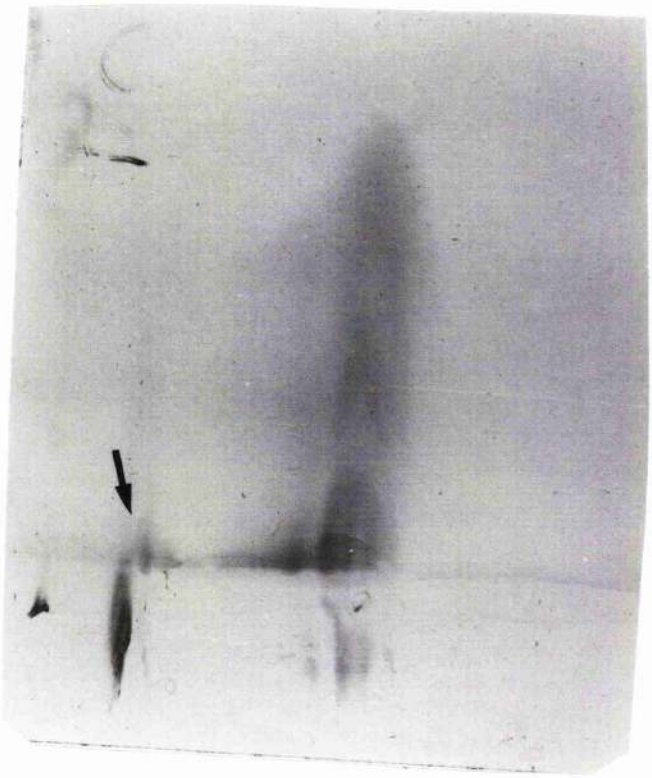


Figure 52 Immunoelectrofocusing of α_2M fractions from multiple sclerosis patient.

8. DISCUSSIONS

Although cystic fibrosis is known to be inherited as an autosomal recessive trait, the basic product of the defective genes still needs to be identified. Current evidence suggests that a genetic defect in an autosomal recessive disease is responsible for a change in a single enzyme or other protein in the body and that a specific clinical or biochemical phenotype, in this type of inheritance, may be produced by more than a single genotype. The phenomenon where a phenotype is produced by more than one genotype is termed genetic heterogeneity. Genetic heterogeneity may result from allelic or non-allelic mutations; that is, the common phenotype may be produced by more than a single mutation at one locus (allelic) or by mutations at different loci (non-allelic). Allelic mutations may be homo-allelic or hetero-allelic. In homo-allelic mutations, different amino acid substitutions occur at the same point in the polypeptide while in hetero-allelic mutations, substitutions occur at different points in a polypeptide chain. To date, no single lesion has proved to provide a unifying hypothesis for the pathogenesis of cystic fibrosis. If more than one allele is involved, definition of the basic defect will become further complicated. Many abnormalities such as pulmonary disease, gastrointestinal disturbances, pancreatic insufficiency and high concentrations of sweat electrolytes have been described, yet, most of these may be several steps removed from the underlying defect(s). Knowledge of the altered gene product is needed, not only for biochemical and immunological diagnosis of heterozygotes and homozygotes but also for guiding the way for rational therapy. To date, only some of the symptoms can be treated or alleviated.

Probably the most intriguing feature of cystic fibrosis concerns the effects of fluids from patients on cilia. The feature was first described by Spock and coworkers in 1967 after observing that serum samples from C.F. patients caused irregularities in ciliary movements of rabbit treacheal explants. Since then the presence of factors in serum and other body fluids from cystic fibrosis patients has been demonstrated by:

(a) Showing that serum and secretions from exocrine glands of cystic fibrosis homozygotes inhibited the reabsorption of sodium that normally would occur in the rat parotid gland (Mangos and McSherry, 1967).

(b) Isoelectric focusing of whole serum in thin layer polyacrylamide gels (Wilson et al, 1975). An extra double protein band was observed between pH8.4 and pH8.6

(c) Isoelectric focusing followed by immunoelectrophoresis (Manson and Brock, 1980).

The basis for the present investigation arose from the reports that a cystic fibrosis factor-like protein could be demonstrated by isoelectric focusing, in thin layer polyacrylamide gels, of whole serum and the IgG fraction thereof of individuals homozygous and heterozygous for the cystic fibrosis gene (Tuly et al, 1979). This study involved electrophoretic analyses in an attempt to detect a C.F. serum factor. The biophysical assay technique of Wilson et al (1977) was reproduced. In addition, extensive investigations using a two-dimensional electrophoresis technique and selective labelling were undertaken. The standardised biophysical assay of Wilson et al (1977) as reproduced in our laboratory enabled the detection of cystic fibrosis protein at pH8.5, but in only 40% of the patients and 20% of the normal controls. Tuly et al (1979) and Wilson et al (1977)

detected the protein in 90% and 100% of the homozygotes respectively. The discrepancy between our results and their reports (Table VI) is not very clear, perhaps since IgG levels were not determined some samples might have been under-loaded. This presumably led to failure of detecting the cystic fibrosis protein. Selective labelling of proteins that focused in the pH8-9 range by the chloramine-T method and then running the labelled proteins in a second dimension SDS gel greatly improved detection. The results we obtained were not able to discriminate cystic fibrosis samples from normal control ones. Assuming that the cystic fibrosis factor detected by isoelectric focusing (Wilson et al, 1976) is identical to the ciliary inhibitor characterised by Bowman et al (1975), then the cystic fibrosis protein would be expected to have molecular weight between 1000 and 12 000, be positively charged and heat labile (Bowman, 1975; Conover et al 1974). Our radiolabelling procedure detected positively charged paptides with molecular weights less than 9 000, but both normal control samples and cystic fibrosis samples contained these low molecular weight peptides. Densitometric scans in an effort to quantitate the peptides in this region were consistent but no differences were detectable between cystic fibrosis samples and those from normal controls (figures 28 and 29). It has been postulated that the cystic fibrosis factor might be C3a (Conod and Conover, 1973), an anaphylatoxic component of the immune complement system. C3a has a molecular weight of 8 900, is very basic and is inactivated by a peptidase that removes the C-terminal arginine. A deficiency of this enzyme might presumably account for the presence of cystic fibrosis factors. Interestingly, investigations in some laboratories have shown that plasma from cystic fibrosis homozygotes had one half as much arginine esterase activity as did control plasma.

The most important objective in cystic fibrosis research, at least from a genetic point of view, is the identification of a dependable genetic marker. At present none of the identified biochemical abnormalities of the disease is clearly related to the symptoms and it is not at all clear which of the symptoms are nearer to the genetic defect(s). An important goal for genetic research should be that all genotypes be clearly distinguishable and any valid tests developed for screening and genetic counseling be specific, reproducible, simple and inexpensive. We, here attempted to find genetic markers of cystic fibrosis by analysing serum samples by two dimensional electrophoresis technique first described by O'Farrell (1975) and adapted by Anderson and Anderson (1977). This technique has two principal advantages:

(I) it deals almost exclusively with individual gene products, thus simplifying genetic analysis of variants,

(II) it combines two very different high resolution separations giving a combined resolution better than 0.1 charge unit and 1 000 daltons for a protein of average molecular weight of 50 000. The protein patterns we obtained, as shown in our results, were basically the same for cystic fibrosis homozygotes and normal controls. Genetic variants of albumin, haptoglobin α and β chains, α_1-anti trypsin, transferrin and fibrinogen, to name but a few, were observed but none of these seemed to be related to the disease under investigation. Since the proteins were first solubilised in 2% sodium dodecyl sulphate/5% 2-mercaptoethanol and boiled for 5 minutes and then brought to 9.5 murea/10% NP-40, hidden charge change mutations were unlikely. Although our results did not show different protein patterns for cystic fibrosis and normal control samples, we could not rule out:

(i) the presence of mutations not accompanied by changes in isoelectric points or molecular weights,

(ii) the presence of charge-change mutations but with the mutant protein migrating to the same point as an abundant protein that obscured it,

(iii) the presence of mutant protein too small to be recognised and resolved in the 7-24% SDS gel used.

It is possible that a mutant protein might have been present in samples from cystic fibrosis patients but co-migrated with ampholines that also stained with Coomassie blue.

What exactly the cystic fibrosis protein is, or what it does in the patient or heterozygote or what it derives from is still unsolved. Even though it has been demonstrated in many body fluids and tissues in cystic fibrosis suffers, this protein is generally believed not to be the primary product of the defective gene. The protein is believed to be a by-product of metabolism formed either

(a) only in people carrying the defective gene i.e. homozygotes and heterozygotes, or

(b) in both patients and normal subjects but not broken down further in homozygotes and heterozygotes. Since proteases are responsible for breaking down proteins, this may suggest abnormalities in either proteases themselves or protease inhibitors. Recently, Nadler et al (1980) have reported that MUGB-reactive proteases in plasma of cystic fibrosis patients and amniotic fluid surrounding CF fetuses were defective. (MUGB = methyl umbeliferyl guanadino benzoate). This finding, though needing confirmation, has a great potential, not only for confirming CF sufferers but for prenatal diagnosis of cystic fibrosis patients.

Plasma protease mediators of blood clotting, fibrinolysis, complement generation and kinin production share a common framework of enzymatic activation sequences and inhibition by plasma protease inhibitors. Abnormalities in these proteases or in the function of their plasma inhibitors might logically be expected to produce alterations in tissue membrane functions and permeability. Defective transport of ions across the membrane is but one of many processes that may be affected by such membrane alterations. The two main protease inhibitors in plasma are alpha-1-antitrypsin and alpha-2-macroglobulin. Much evidence exists today that alpha-1-antitrypsin is quite normal in cystic fibrosis (Romeo et al, 1980). Our two dimensional electrophoresis results produced variants of alpha-1-antitrypsin but these were not related to cystic fibrosis. Reports on the other main protease inhibitor, alpha-2-macroglobulin, are conflicting. It was reported that in most cystic fibrosis patients, an α_2M-proteinase complex was missing (Shapira et al, 1976) but recent reports (Romeo et al, 1980) have failed to confirm this and also two dimensional electrophoresis of purified α_2M was not able to detect mutant forms in cystic fibrosis (Comings et al, 1980). Our results were not able to distinguish α_2M preparations from cystic fibrosis patients from those prepared from normal control subjects as analysed by:

(I) SDS gel electrophoresis of heat induced fragments.

(II) Two dimensional gel electrophoresis

and (III) Immunoelectrofocusing.

In this laboratory, previous experiments in which α_2M preparations were digested with trypsin and the products analysed by SDS gel electrophoresis, were not able to show any differences between preparations from cystic fibrosis and normal controls (Burdon, 1980).

Concluding Remark:

Although our investigations of cystic fibrosis serum were very extensive and results fully convincing, we still remain open to the unexpected.

Future Trends:

(I) The central problem still remains that of identifying the genetic markers and then developing objective and reliable methods of measuring them.

(II) Finding logical relationship between observed biochemical abnormalities and symptoms and then developing rational therapy would be very beneficial to the affected individuals.

(III) One of the outstanding questions requiring answering is whether cystic fibrosis is a single disorder or a group of disorders.

(IV) Almost all cystic fibrosis patients develop infections of the respiratory system. Information on host defense mechanisms against bacterial invasions is required.

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